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A STOMACH LESION IN GUINEA-PIGS CAUSED BY DIPHTHERIA TOXIN AND ITS BEARING UPON EXPERIMENTAL GASTRIC ULCER.*

M. J. ROSENAU AND JOHN F. ANDERSON.

(From the Hygienic Laboratory, Public Health and Marine Hospital Service.)

GUINEA-PIGS dying acutely from injections of diphtheria toxin frequently show a lesion in the stomach which, so far as we know, has not been described.

Our attention was first called to the condition of the stomachs of guinea-pigs used in our diphtheria work by Assistant Surgeon A. M. Stimson of the Public Health and Marine Hospital Service, in the summer of 1905. Since that time we have collected from our autopsy records 2,882 protocols in which the condition of the stomach was noted. Of these 1,897 guinea-pigs, or 66 per cent, showed the lesion in the stomach described in this paper.

In the many thousand autopsies upon guinea-pigs used in this Laboratory in preparing the standard unit for measuring the strength of diphtheria antitoxin and also for verifying the potency of the antidiphtheric serum made by licensed manufacturers, we constantly see the lesions usually found in animals dying from diphtheria poisoning, such as darkened and congested adrenals, effusions into the

* Received for publication August 15, 1906

pleuræ, and other serous cavities, local edema, necrosis and hemorrhagic reaction at the site of inoculation, etc.; but it is difficult for us to understand how the condition of the mucosa of the stomach, which is so very evidently present in such a large proportion of the guinea-pigs, has escaped the observation of others.

This lesion may assume practical importance in view of the light it may throw upon experimental gastric ulcer.

The experimental production of gastric ulcer, has met with little success. The following table of experiments by previous investigators, taken from Turck's article[1] upon ulcer of the stomach gives in condensed form the method used and results obtained.

Turck, in following the subject further, has succeeded in producing ulcers of the stomach and duodenum in dogs by feeding *B. coli* for a variable length of time and as a result of his work considers that we now have, for the first time, a firm basis upon which to solve the final or underlying etiology of gastric ulcer.

In connection with the lesion in guinea-pigs which we describe it is interesting to note that Turck attempted the production of gastric ulcer in dogs by the injection of diphtheria toxin into the stomach wall. This, it seemed, did not produce an ulcer, but pinhead hemorrhagic foci in the duodenum or local necroses near the pylorus. When the diphtheria toxin is injected into the mesenteric vessels Turck also failed to produce ulcer, but obtained necroses in two weeks in the duodenum and near the pylorus.

We find that gastric ulcer may be produced in the guinea-pig by the subcutaneous inoculation of diphtheria toxin given in sufficient quantity to cause the acute death of the animal. It follows injections of a minimal lethal dose, as well as the injection of the toxin-antitoxin mixture (L+ dose) used in measuring the strength of diphtheria antitoxin. When the toxin is completely neutralized by the antitoxin, as in the case of the L° mixture, and the animal afterward killed, the stomach shows no lesion.

The lesion is caused by infection with the diphtheria bacillus as well as injections of the diphtheria toxin. We injected a number of guinea-pigs with a young agar culture of *B. diphtheriae* well washed to free it from toxin. The animals sickened and died in from four to

[1] *Jour. Am. Med. Assoc.*, 1906, 46, p. 1753.

TABLES OF EXPERIMENTS BY PREVIOUS INVESTIGATORS.

Investigator	Method	Result
A. Mechanical and Physical Injury—		
Ritter	Violent bruises	
Decker	Heat	Ulcer
Matthes	Trauma (with 5 per cent HCl)	Ulcer
Schmidt	Trauma (with HCl)	
Körte	Pinching stomach with clamps	Ulcer
B. Chemical—		
Roth	Crystals of nitrate of silver introduced into stomach	Ulcer
1. HCl as a necessary factor—		
Riegel	HCl necessary	
Matthes	Trauma (without HCl)	Negative
	Trauma (with 5 per cent HCl)	Ulcer
Schmidt	HCl and trauma	
Ewald	HCl essential factor	
2. Contra HCl—		
Pawlow	Hyperacidity a consequence	
Du Misinl	Superacidity without significance	
Ageron	HCl may be persistently absent	
Kavetsky	Synchronous ulcer of the stomach and bladder	
C. General Dysemia—		
Virchow	Anemia and chlorosis	
Quincke and Daetwyler	Anemia by producing gradual hemorrhage and local trauma	Ulcer which healed with difficulty
Silbermann	Hemoglobinemia	Ulcer
Futterer	"	Ulcer
Cohnheim	" by mechanical injury and injection of laked blood	Ulcers
D. Disturbance of Local Circulation—		
Virchow	Embolism, thrombi, aneurism or varicose veins	
Klebs and Welti	Thrombi	
Panum	Injection of emulsion of wax into femoral vein	Gastric infarcts, ulcer
Talma	Ligation of esophagus and pylorus	Ulcer
Rindfleisch	Venous stasis; prolonged ischemia	
Axel Key	Prolonged ischemia due to contraction of gastric muscle	No experiments
Müller	Tied portal vein	Ulcer
E. Injuries to Nerves and Nerve Centers—		
Schiff	Intersection of thalami and peduncles	Ulcer
Ebstein		
Brown-Sequard	Anterior corp. quadrigemina	Ulcer
Vidova	Injection of alcohol into vagus	Negative
Vidova	Section of the sympathetic	Ulcer
Yzeren	Section of the sympathetic with section of vagi below the diaphragm	Ulcer
Saitta	Section of the sympathetic with section of vagi below the diaphragm	Ulcer
Ophüls	Section of the sympathetic with section of vagi below the diaphragm	Ulcer
Koch and Ewald	Section of cord and 5 per cent HCl in stomach	Ulcer
F. Local Infection—		
Cohnheim	Injection. Infected suspensions of lead chromate	Erosions and ulcers
Böttcher	Infection as cause of ulcer	Opposed by Körte
Nauwerck	Infection observed at edge of ulcer	

six days. The autopsy findings, so far as the stomach was concerned, were precisely similar to that seen in animals dead of toxin injections.

So far as we know a similar condition of the stomach in human diphtheria has not been noted.

The lesion in the stomach of the guinea-pig consists of a sharply defined area of congestion, hemorrhage, or ulceration near or at the

pyloric extremity. Occasionally the lesion extends half an inch into the duodenum and in a few instances the duodenum itself shows slight injection or congestion while the stomach remains normal.

The first stage consists principally of a congestion, always in the neighborhood of the pylorus. This is best seen in guinea-pigs dying as a result of overpowering doses of diphtheria toxin in about 24 hours. When the guinea-pig dies later, for instance on the second, third, or fourth day, the lesion is more advanced and consists of hemorrhages into the mucosa followed by destruction of tissue, some-

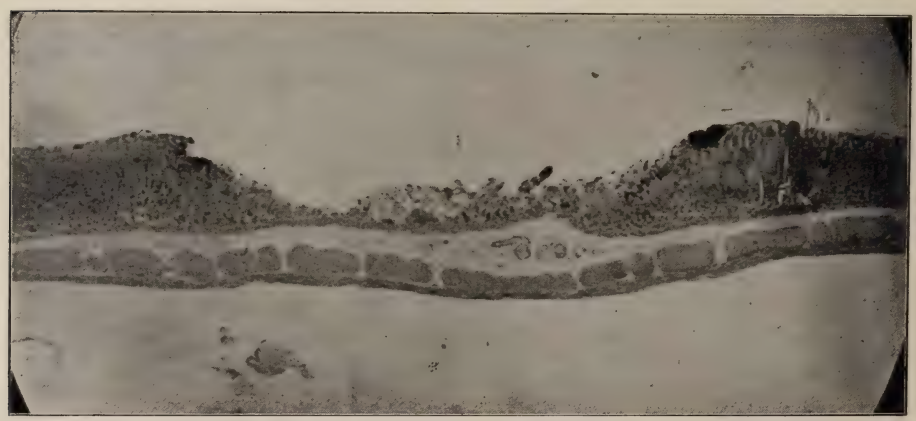

Fig. 1.—Ulcer. Note the absence of cellular infiltration, including digestion of mucosa.

times extending into the muscularis, but in no case has perforation been observed.

It is interesting to know whether guinea-pigs that received less than a lethal dose of the diphtheria poison from which they ultimately recover may have had this stomach lesion. In a few instances in our guinea-pigs which died on the 22d and 28th days with paralysis we found old blood-stained areas in the mucosa of the stomach in the region of the pylorus indicating a healed area.

This lesion is not the result of any particular diphtheria toxin, as we have found that it follows the injection of all the various diphtheria toxins, some 25 in number, used in this laboratory.

There is no relation between the source of the guinea-pig and the effects of the diphtheria toxin, for we have found this lesion in guinea-

pigs obtained from four outside sources as well as those raised in the laboratory.

We have examined a number of healthy guinea-pigs from our stock in order to assure ourselves that no other cause than the diphtheria toxin could possibly account for the appearances that we find in the stomachs of these animals.

It will be seen from the following table that when guinea-pigs are given an injection of diphtheria toxin sufficient to kill them within 24 hours, about half the animals show this lesion. When they die on the third or fourth day a very much larger proportion of the animals show the lesion. About 75 per cent of the guinea-pigs dying between the fourth and fifth days show this lesion; but in guinea-pigs dying later than the 10th day the relative number of animals showing this stomach lesion gradually diminishes. Guinea-pigs dying of late paralysis practically never showed the stomach lesion.

THE FREQUENCY WITH WHICH GUINEA-PIGS, DEAD OF DIPHTHERIA TOXIN, SHOW THE STOMACH LESION.

Time of Death	Condition of Stomach	No.	Per Cent Lesions
Less than 1 day	lesion	7	54
	normal	6	
Between 1 and 2 days	lesion	104	62
	normal	61	
" 2 " 3 "	lesion	833	77
	normal	235	
" 3 " 4 "	lesion	725	73
	normal	254	
" 4 " 5 "	lesion	163	52
	normal	144	
" 5 " 6 "	lesion	35	28
	normal	85	
" 6 " 10 "	lesion	28	25
	normal	84	
More than 10 days	lesion	2	1.7
	normal	116	
Totals	Stomach lesion	1,897	Average: 66
	" normal	985	
		2,882	

CONTROLS.

A number of comparisons were made with tetanus toxin and various chemical poisons, but in no instance was there found any similar sharply defined lesion in the pyloric extremity of the stomach.

The stomachs of a number of guinea-pigs dead of tetanus toxin appeared quite normal.

The stomachs of guinea-pigs dying acutely from strong poisons, such as hydrocyanic acid, chloralcyanhydrin, etc., sometimes show acute hemorrhagic areas either of a diffuse or punctate nature, but in such cases the lesion is not confined to the pyloric end of the stomach.

For the effect of various chemical poisons upon the stomach of guinea-pigs we are indebted to Dr. Reid Hunt, Chief of the Division of Pharmacology, Hygienic Laboratory, for the following data upon animals used by him in other lines of research and which serve as our controls:

Hydrocyanic acid.—Five guinea-pigs were given subcutaneously from 0.005 to 0.006 mg. of hydrocyanic acid, per gram of guinea-pig, which caused death in 1 hour 15 minutes to 6 hours 25 minutes.

G.-P. No. 315 (0.005 mg.). Mucosa and submucosa of anterior and posterior walls of stomach showed large acute hemorrhagic areas. Pylorus apparently normal.

G.-P. No. 305 (0.0052 mg.). Stomach showed intense hemorrhage into the mucous membrane. Pylorus apparently normal.

G.-P. No. 340 (0.0055 mg.). Mucous membrane of whole of stomach and submucosa intensely hemorrhagic.

G.-P. No. 327 (0.006 mg.). Enormous punctate hemorrhagic areas on mucous membrane of greater curvature of stomach.

G.-P. No. 365 (0.005 mg.). Mucosa of stomach slightly injected and hemorrhagic.

Chloralcyanhydrin.—Eight guinea-pigs were injected subcutaneously with chloralcyanhydrin in quantities varying from 0.0052 to 0.02 mg. per gram, which caused the death of the animals in from 6 hours to 16 minutes. The stomach in each case was normal excepting in the two animals receiving the large amounts, as follows:

G.-P. No. 330 (0.025 mg.). Acute hemorrhage of the posterior and inferior walls of the stomach, involving both the mucosa and the serous coats.

G.-P. No. 325 (0.03 mg.). Stomach shows numerous hemorrhagic areas.

Strophanthin.—Four guinea-pigs were injected subcutaneously with strophanthin in amounts varying from 0.001 to 0.005 mg. per gram, causing the death of the animals in 1 hour 35 minutes to 43 minutes. In each case the stomach appeared normal.

Strychnin sulphate.—Ten guinea-pigs were injected subcutaneously with strychnin sulphate, and others with strychnin sulphate plus alcohol. The amount of strychnin varied from 0.004 mg. to 0.005 mg. per gram, which caused the death of the animals in 11

PLATE 1.

FIG. 1.

FIG. 2.

minutes to 4 hours 20 minutes. The stomach was normal in each instance.

Nitroprussiate of soda.—Three guinea-pigs were injected with amounts of nitroprussiate of soda varying from 0.015 to 0.018 mg. per gram, which caused the death of the animals in 1 hour 15 minutes to 2 hours 15 minutes. The stomach was normal in each instance.

EXPLANATION OF PLATE 1.

FIG. 1.—Stomach of guinea-pig showing mild grade of diphtheria lesion.

FIG. 2.—Stomach of guinea-pig showing severer grade, with hemorrhages and ulceration. Note the sharp line of demarkation and limitation of the pyloric extremity.

NOMA.*†

(GANGRENOUS STOMATITIS; WATER CANCER; SCORBUTIC CANCER; GANGRENA ORIS; GANGRENE OF THE MOUTH.)

GEORGE H. WEAVER AND RUTH TUNNICLIFF.
(From the Memorial Institute for Infectious Diseases, Chicago.)

INTRODUCTION.

TOURDES has defined noma as a gangrenous affection of the mouth especially attacking children in whom the constitution is altered by bad hygiene and serious illness, especially from the eruptive fevers, beginning in an ulcer of the mucous membrane with edema of the face, extending from within out, rapidly destroying the soft parts and the bone, accompanied most often by hepatization of the lungs, and almost always quickly fatal. Other writers have included under this head all of the gangrenous processes which involve the skin of children. There has been much difference of opinion as to the relationship borne by various ulcerative processes in the mouth to noma. Some of the studies carried on during the past few years seem to point to a common etiology for many of the pseudo-membranous and ulcerative lesions of the naso-pharyngeal mucous membrane and for noma, the difference in the degree of destruction of tissue depending upon varying degrees of vulnerability of the tissues, and perhaps in a less degree upon varying degrees of virulence of the bacteria concerned.

In the following article we shall use the word "noma" as a term to cover all these forms of gangrene which involve the mucous membrane and skin with the intervening isssues in the neighborhood of the orifices of the body.

HISTORICAL SKETCH.

Such a striking and fearful disease as noma could not fail to be noted by the first physicians who came in contact with it. That this was the case is evident from the abundant literature relating to it collected by A. L. Richter in his publications in 1828, 1832, and 1834. Richter was the first author of a monograph upon noma, and in his first publication he collected 74 references to this disease in the literature. They were gleaned from the various parts of the world. A few of the earlier historical facts connected with the disease are given here, many being quoted from Richter's monograph.

The disease was known to the most ancient medical writers, such as Hippocrates, Galen, Celsus, Arethaeus, Caelius Aurelianus, and Alexander de Tralles. Hippocrates applied the name noma to all kinds of gangrene and ulcers, and connected it especially with putrid decomposition and considerable destruction of tissue. Galen mentions noma in many passages of his work. He defines it as an affection characterized by destruction of tissue extending from the diseased parts to the healthy. He also connected noma and putrid decomposition. He classed together noma and

* Received for publication, May 1, 1906.

† In the study of the case of noma herein reported, a review of literature covering many years was undertaken. With the hope of sparing some of this labor to those who in the future may have occasion to study such cases, an effort has been made to incorporate in this article the most important facts bearing upon the subject.

the affections of the mouth, anus, and genitalia. He recommended for treatment aromatics, stimulants, and caustics, and recognized the necessity of the red-hot iron. Celsus, who is credited with giving the most precise description, according to Tourdes, was not describing noma but malignant pustule. Arethaeus described an aphthous ulceration which had many analogies to the gangrenous affections of the mouth. Caelius Aurelianus and Alexander Trallianus both used the term noma with the idea of putrid decomposition. Noma was probably rarer then than later, because the conditions predisposing to it were less prevalent. The absence of hospitals and the eruptive fevers account for its infrequent occurrence in ancient times, according to Tourdes.

Baltus, in 1620, a Dutch surgeon, furnished the first accurate description of gangrene of the mouth in his *Handbook of Surgery*. Van de Voorde (1662), introduced the name "water-kanker," which was usually employed by subsequent Dutch writers. Van Swieten gave it the name "gangrene." J. Van Lil called it "ulcus noma, stomacace, and water-kanker." He referred to many Dutch authors who observed epidemics of the disease following exanthematous diseases. Most of the Dutch writers pointed out the influence of scurvy on this condition.

Swedish physicians were also familiar with the nature and course of the disease. Lund based his writings on 11 cases, only one of which recovered. He recognized the fact that it occurred only among the children of the poor who lived in damp, unhealthy atmosphere and were poorly nourished. Leutin described the disease most accurately under the name of "ulocace."

In England, Boot first described the disease. Subsequent writers in England were Underwood, Symmonds, Pearson, S. Cooper, Marshall-Hall, West, and others.

In America the disease was early described by Coates, Gerhard, Meigs and Pepper. Coates, writing in 1826, states that ever since the establishment of the Children's Asylum in Philadelphia, in the spring of 1819, the institution had been annually visited by the new and distressing scourge. He further says: "It has here prevailed in a considerable number of cases, forming the principal source of anxiety and trouble during the winter season, and annually sweeping off its little victims in a manner rendered peculiarly awful by its insidious approach, its loathsome effects, and its apparently uncontrollable progress." He observed 70 cases among 240 children, but from his description he evidently included cases of ulcerative stomatitis. Coates says the disease was rare in Philadelphia, but quite prevalent in Salem, N. J. He refers to other American authors who had observed cases of the disease.

German physicians have furnished the most and best monographs upon this disease. Preceding A. L. Richter it was carefully studied by Fabricius von Hilden under "de catarrho ad gingivas," by A. G. Richter, and by Jawandte under "noma or gangrene of the mouth." Wendt recognized it as the most fearful sequel of scarlatina and measles, and called it "sphacelus" of the mouth. C. F. Fischer and Siebert sought especially to turn attention to this disease. The latter believed a scorbutic disposition essential for its development, and emphasized the value of local remedies, especially acids. Hildebrand, Girtanner, Fleisch, Feiler, Henke, Jörg, and Raimann held that water cancer is the latest stage of the symptoms belonging to scorbutus. Raimann discarded the name cancer, and proposed that of very acute, pultaceous or caseous gangrene. Wiegand and Klaatsch undertook to differentiate between water cancer and scorbutus. In 1828 A. L. Richter published the first monograph on noma, under the title of *Wasserkrebs*, and in 1832 he completed it by a supplement. His

collection of the literature up to that time is so complete that subsequent writers have found it needless to go back of his work.

In France the disease had early received much attention. Poupart and Saviart observed gangrene of the mouth in Hôtel-Dieu. Berthe described it under the name, "scorbutic gangrene of the gums" ("gangrène scorbutique des gencives"). Sauvages described it under "necrosis infantilis." Baron in 1816 published a description of the disease, and concluded that gangrene of the mouth is an affection *sui generis*, and local. He found the best treatment, if perforation has occurred, to be the hot iron. In 1818, Isnard, in his inaugural thesis, described gangrene of the mouth and vulva. He favored the use of the actual cautery after excision of gangrenous tissue. Some less important publications by Cliet, Rey, Destrées, Billard, Murdock, Boeckel, Constant, Taupin (1839), and others, were followed by the very complete monograph by Tourdes in 1848. Bouley and Caillault published a valuable contribution in 1852. This brings us up to the publication of the most comprehensive description by Barthez and Rilliet in 1855. Among the more recent noteworthy and exhaustive writings upon noma may be mentioned those of Bruns in 1859, Gierke in 1868, Hirsch 1862–64, and Bohn in 1880.

Bruns says that Uzanam in 1823 describes the disease occurring in Spain, as "fegra," "fegar, "or "fegarite." In 1810 it occurred among the French troops in Madrid, attacking more than 100. It was said to occur among children in Spain, appearing to be contagious. Gangrene of the mouth appears to have been rare in Italy. Benevoli, Bartholomeo, Maggi, and Joanès Togault are mentioned by German writers as contributing articles on the subject of noma.

FREQUENCY OF OCCURRENCE.

Noma is an uncommon disease. In private practice it is almost never met with, and the experience of Meigs, who "never met with a case in private practice," corresponds with that of most practitioners. Even in hospitals the disease is uncommon. Allbutt says that only one case appeared on the records of Westminster Hospital in 10 years. According to Allchin, at the East London Hospital for Children, which is situated in a very poor and densely crowded district, during seven years, 1881–87 inclusive, only five cases occurred in a total of 6,364 admissions during that time. At Great Ormond Street Hospital, during 13 years, 1876-88, only six cases occurred, with a total of more than 13,000 patients.

In some continental hospitals the proportion of cases seems larger. Gierke states that during 16 years, 20 cases were observed in the Children's Hospital of Stettin. In the Children's Hospital in Prag, according to Springer, 65 cases occurred in 30 years from 1857 to 1887, and 23 cases in the 16 years from 1888 to 1903. Woronichin saw noma occur in 22 out of 8,286 hospital patients, and in 24 out 207,259 ambulatory patients.

ETIOLOGY.

Age.—Noma is a disease which occurs almost exclusively in young children. The largest number of the cases are observed between the second and seventh years of age, a few being seen in the first year, and occasional ones up to the twelfth. According to Tourdes many physicians believed that immunity was acquired against the disease during the period of lactation. This author (1848) reported nine authors who had seen noma in persons between 15 and 72 years of age. Bruns in 1859 referred

to 11 authors who had observed cases in persons between the ages of 15 and 70. In 1872 Strueh reported a case in a man 30 years old. It was unusual in that it could not be traced to any other disease, the patient apparently being in good health when the gangrenous process began. He referred to a case observed by Vogel in a patient 18 years of age also with no apparent cause. Masterman in 1891 recorded two cases in adults in which no cause could be found. In 1873 Hildebrand (quoted by Ziegler) collected 22 cases from the literature in persons over 15 years of age. In most cases it followed acute infectious diseases, especially measles. Other more recent authors have reported cases in adults following infectious diseases: Brydon, after erysipelas of the leg, at 47 years; Zieger, at 28 years; Köster, following hemorrhagic pleuritis; LeCount, following amebic dysentery; Kraus, after typhoid fever. Herff, under hospital gangrene of the vulva, and Herman, under acute gangrene of the vulva, have reported cases which perhaps belong in the same group.

Sex.—It has been said by Tourdes and others that noma is more frequent in girls than boys. This is explained by the inclusion in the statistics of cases of noma of the genitalia, this location of the disease being observed quite frequently in girls. Girls do not appear to be more susceptible, but present greater opportunities for local predisposing conditions.

Hygienic conditions.—Most authors have laid much stress upon a constitutional condition which must be present in order for the disease to occur. Richter believed the cause must be sought in the organism of the child. Barthez and Rilliet say that it usually occurs in weakly children. Gierke's cases all occurred in poorly nourished anemic children from poor families. Mayr, writing of noma cases accompanying or following measles, insisted that depressing conditions other than measles are always necessary. According to Barthez and Rilliet, it sometimes occurs in strong children. Strueh referred to four cases reported by Rust, Siebert, and Klaatsch, in well nourished children, without preceding sickness. Cases in apparently healthy adults have already been referred to.

Noma has generally been said to be a disease of the children of the poor, and is apt to occur in conditions of uncleanliness, over-crowding, damp, bad air, and poor food. Boeckel (cited by Tourdes) reported a case in a child living under the best hygienic conditions. Gierke noted that it might occur in good surroundings in poorly nourished children in the course of certain diseases. Coates's cases occurred in a hospital hygienically located, and the epidemic reported by Blumer and MacFarlane occurred in a modern hospital in which the general hygienic and dietetic conditions were excellent. The occurrence appears to be dependent more upon the individual than upon surroundings, except as the latter, if bad, may lower the resistance of the individual, and favor the development of weakly children.

Climate.—That climate may play a part has been pointed out by Tourdes and Barthez and Rilliet, who say that the disease is endemic in cold and damp climates, as Holland, Sweden, and the Prussian coast. However some of the larger groups of cases have been observed in inland cities. Tourdes mentions its presence in marshy countries.

Season.—It is usually stated that the cases occur most often in the winter, rarely in the summer. Tourdes says that it is most prevalent in the spring and fall. Gierke observed it throughout the year, but more in the winter, while Mayr says that the season is without influence. In Woronichin's series of 22 cases, about as many occurred in the summer as in the winter. The greater prevalence of epidemics

of measles during the winter, may serve to account for the larger proportion of cases occurring at that season.

Pathological influences.—All writers agree upon the important predisposing part played by chronic and acute diseases. Baron says that gangrene of the mouth is never primary, but occurs in children weakened by a former disease. Barthez and Rilliet agree with this entirely. It has been observed in connection with, or as a sequel of, measles, scarlatina, variola, pneumonia, pertussis, intestinal diseases, intermittent fever, diphtheria, typhoid fever, syphilis, varicella, erysipelas, rhachitis, hemophilia, tuberculosis, pulmonary gangrene, bronchial catarrh, and amebic dysentery. Tourdes analyzed 98 cases observed by various authors and found the order of frequency of the different diseases to be as follows:

Measles	39	Pulmonary tuberculosis	3
Intermittent fever	8	Pneumonia	2
Typhoid fever	7	Dysentery	2
Mercurialism	7	Scurvy	2
Pertussis	6	Syphilis	2
Scarlatina	5	Bronchitis	1
Variola	5	Diphtheria	1
Enteritis	5	Cerebral congestion	1
Scrofula	4		

Of these predisposing diseases measles stands pre-eminently first. Osler claims that at least one-half of the cases develop during the convalescence from measles. Krahn records 33 out of 133 cases following measles. Bouley and Caillault found measles was the antecedent in 41 out of 46 cases. Allbutt places typhoid fever in the second place. Carrière-Montjosieu reports 11 cases of noma following typhoid fever and says it is not a rare complication. Keen (quoted by Sailer) found noma recorded in nine out of 1,700 cases of typhoid fever with surgical complications. Cases following scarlatina are uncommon. Tourdes recorded only five out of 98 cases, and Woronichin only four out of 22 cases. The case referred to in this article followed this disease.

Taupin believed that there was almost an antagonism between noma and tuberculosis, but this was not proved by autopsies done later by Barthez and Rilliet. Barthez and Rilliet say that noma rarely occurs in cases of extreme tuberculosis, but in nine out of 20 sections upon noma cases they found slight tuberculosis lesions, which led them to assume a connection between gangrene and the after-products of the tubercle.

The evidence that scurvy was a great etiological factor in noma, as was believed by the ancient physicians, was much exaggerated, according to Tourdes, who says it has not been found by modern observers to be associated with this disease.

According to Perthes (1902) noma is associated with splenic tumor developing after malaria.

Therapeutic influences.—It was formerly supposed that calomel had much to do with the causation of the disease. Dieffenbach and Simon, cited by Tourdes, affirm positively to having seen noma follow large doses of calomel. Gierke and Mayr think it may favor the occurrence of noma in a few cases, but they, as well as Coates and West, deny that it plays any considerable rôle.

Contagiousness.—There are few or no examples of well-authenticated contagion, although a few instances are at least very suggestive. Bruns refers to an instance observed by Lund in which a case developed in a room in which a sister had died of the same disease; and to an observation by Siebert, in which two sisters and two

other patients were attacked in the same house. Mayr believed in the contagiousness of noma and insisted on isolation. Holt considers there is little doubt as to the contagiousness of noma. He observed five cases, following whooping-cough, develop in a single ward, all beginning in the auditory canal. They were apparently produced by using the same syringe which had not been properly disinfected. Most writers have denied the occurrence of contagion, and have shown that it did not occur even when abundant opportunity was presented. The question must be considered unsettled, but with the present tendency to ascribe to certain bacteria the etiological rôle, the possibility of direct transfer becomes most important, and even if certain saprophytic bacteria are the active factors, in their condition of heightened virulence they may be better able to infect other persons than when in their normal condition.

Epidemics of noma have been described. Barthez and Rilliet never observed an epidemic. Bouchut says that it sometimes occurs epidemically. The occurrence of epidemics, like that of contagion, is doubted by most writers. Blumer and MacFarlane in 1901 reported an epidemic of noma in the Albany Orphan Asylum, during which 16 cases developed among 173 children who had suffered from measles during a severe institutional epidemic. The type of the measles was severe, often accompanied by complications. Of the 16 cases reported, all occurred in the girls' dormitory, except two cases in boys in the infirmary, where cases of gangrene were being treated. Although there were nearly twice as many cases of measles among the boys as among the girls, no case of noma developed in their dormitory. After the cases were thoroughly isolated no new cases developed.

Bacteriology.—The bacterial study of noma has always been conducted with certain difficulties, one being the rarity of the disease, so that a series of cases have not come under the observation of any one investigator within a reasonably short time; another being the large number of bacteria found in the gangrenous tissue, some of which cannot be grown by aerobic methods or upon the nutrient media usually employed; and a third being the difficulty of producing a corresponding disease in experimental animals.

There are two varieties of organisms which must be considered as having been shown to be associated with the disease by several independent investigators; the anaerobic, threadlike organism first cultivated by Seiffert, and the diphtheria bacillus.

The threadlike organism, which was first cultivated by Seiffert in 1897, was undoubtedly observed in nomatous tissues before that time. Lingard in 1888 described long, threadlike growths which were found in great numbers at the line of the extension of the necrotic patch. He did not cultivate it nor describe its staining properties. Grawitz in 1890, in a single case, observed bacilli at the junction of the necrotic and healthy tissue, which grew into long threads. No cultures were made. The organisms stained by Gram's method. Bartels in 1892 examined microscopically material long preserved in alcohol from two cases. Deep in the necrotic tissue he found innumerable long, slim bacilli, often appearing as threads. They were most numerous near the demarkation zone, and passed with the loose connective tissue from the necrotic tissue into the infiltrated zone. They stained by Loeffler's method, while the staining by Gram's or Weigert's methods was not always successful. He demonstrated by special stains that the threads were not elastic tissue. Foote in 1893 studied a case in a girl of seven years, in whom whooping-cough followed by typhoid fever had prepared the soil for noma. Along the border of the necrotic zone a bacillus which was often seen in long strings was present in preponderating numbers. It

was not confined to the necrotic area, but had also infiltrated the sound tissue in smaller numbers. It is said to have stained by Gram's method, but that great care was necessary to avoid decolorizing too much. In the older necrotic tissues a variety of cocci were present.

Elder in 1893 studied a case in a girl of four and one-half years, following measles. Aerobic cultures removed soon after death remained negative. In sections long, thin bacilli, tapering at the ends and commonly in pairs, were seen around the vessels and extending outward into the tissue. They were well seen passing between the fat cells of the tissues of the cheek. The organism stained pretty well by Gram's methods but were readily decolorized by clove oil.

Seiffert in 1897 studied two cases of noma, and by means of anaerobic cultures he obtained a cladothrix, which he believed to cause gangrene. The cultures produced a progressive necrosis in guinea-pigs which eventually terminated in suppuration. Suppuration was also produced in rabbits. He was apparently the first one to cultivate this organism.

Schmidt in 1898 studied a case in a child of 7 years. In the boundary zone and in the necrotic tissue were found abundant, delicate bacilli with rounded ends. They were observed to follow the connective tissue into the healthy tissue, and were especially abundant in vessels lying within the necrotic area. In many places were seen bent, delicate threads, apparently consisting of the same bacilli.

Perthes in 1899 studied two cases. In sections of the tissues which were stained 24 hours in carbol-fuchsin and decolorized with 70 per cent alcohol, he found what he designates as a streptothrix. In the necrotic tissue were seen threaded structures in great abundance. The caliber of the threads was variable, the larger being recognized as composed of a line of bacilli, the finest appearing more homogeneous and many winding over the greater part of the field. Some threads exhibited spindle-shaped enlargements. Among the threads were found rods of varying length and many spindle-like elements of varying dimensions. As the living tissue was approached the threads became more rare and the finer ones correspondingly numerous, lying often in thick bundles or tongues and following the direction of the still distinct tissue fibers. The nearer the sound tissue was approached the more the field filled with threads of extreme fineness. Beyond the border of the necrotic tissue in the sound tissue isolated threads were observed lying between the living cells. Here spiral forms were observed. If Weigert's stain was employed, only the larger threads were stained, and even in these, parts did not retain the stain. In teased preparations from the border zone some threads were seen to exhibit dichotomous branching, and strongly bent "spirilla" were also seen. The author believes that the various forms observed in the border zone belong to one variety of bacterium.

In deep agar cultures (anaerobic) a cloudlike growth occurred about the fragments. This growth was always mixed, but contained threads closely resembling those in the tissues, both in form and staining properties. Pure cultures could not be obtained. Because the organism is most abundant in the necrotic tissues, and because it is found among the sound tissue elements, he believed it to be the cause of the disease.

In 1902 Perthes reported the frequent occurrence of noma in the Chinese, associated with the splenic tumor of malaria. In these cases he found the same bacteria he had previously reported in cases in Germany. He insists that "spirilla" are terminal enlargements or stages in the development of fusiform bacilli.

Krahn, in 1900, described in the tissues of a case of noma threads corresponding to those observed by Perthes. He was unable to observe branching, and because of this, and because of the fact that threads were sometimes made up of distinct links, he concluded that the organism was not a streptothrix. The threadlike organisms were not cultivated. His idea is that noma is due to a mixed infection by various mouth bacteria, especially with the "*Spirillum sputigenum*" and "*Spirochaetae dentium*."

Blumer and MacFarlane in 1901 reported a most interesting epidemic of noma, in which 16 cases of noma occurred among 173 cases of measles. In all cases a threadlike organism was present in cover-slip preparations. It stained best with carbol-fuchsin, and stained faintly by Gram's method. In sections it was best seen after staining with carbol-fuchsin and decolorizing in oil of cloves according to Flexner's method. In such sections in the deeper part of the necrotic zone the organism was present in enormous numbers. The individual bacteria were nearly always seen as long threads, which for the most part lay parallel and between the connective tissue fibrillae. The organisms were fewer in the zone of reaction, and a small number were found in apparently unaltered tissue. Efforts to cultivate the organism failed.

Seiffert in 1901 reported the study of four additional cases, in all of which he was able by anaerobic methods to cultivate the same threads, "spirilla," and branching forms of cladothrix as in the earlier cases. By animal experiments he showed his cultures to be pathogenic for guinea-pigs and rabbits, and he observed a spontaneous contact-infection of the lip of a rabbit.

Braun, in 1901, in discussing Seiffert's presentation, referred to a case of noma in which he had found the thread organism described by Seiffert, but efforts to cultivate it anaerobically or transfer it to animals were futile.

In 1902 Matzenauer published a paper upon noma and hospital gangrene in which he attempted to show that the two diseases are identical clinically and bacteriologically. In the nomatous tissues he described, in the border zone, a great number of bacilli of uniform shape, in entangled heaps, staining intensely and uniformly by Weigert's stain. The bacilli were slender, straight, or slightly curved, usually single, often in pairs end to end. The ends were usually not angular but slightly rounded. If the process was progressive the bacilli were deep in the tissues where there was no necrosis or even marked inflammatory reaction, and only fibrin network was present about the vessels. The bacillus is said to be anaerobic, but no details are given as to the cultures. He believes these bacilli are the same as observed by most former authors in cases of noma, and apparently identical with the bacilli observed by Vincent and himself in hospital gangrene.

In 1903 Ranke was ready to declare his conviction that noma was caused by a fungus, closely related to actinomyces. The threads did not stain by Gram's method.

Rosenberger in 1904 reported some observations in a child with noma of the cheek. In smears from the margin nearest the mouth there were various bacteria. Most abundant were "spirilla" and bacilli arranged in pairs, the distal ends being pointed. Cultivation of these bacteria was not accomplished.

Brüning in 1904 described in sections of nomatous tissue, thick tangles of filaments, but in his illustrations he figures only individual bacilli. He was able to grow the same organism as described by Seiffert in anaerobic cultures in bouillon and agar.

Hofmann in 1904 found, in a case of noma, bacilli in the freshly infiltrated part

as well as in the necrotic portions. They were five micra in length, straight or comma-shaped, and were sometimes swollen in the center. Threads two or three times as long were also observed without regard to location. They showed spirils, but never more than two. "Spirilla" were observed in the necrotic tissue. Both the bacilli and "spirilla" were seen in the subcutaneous fatty tissue. The spirilla were similar to those found in the mouth. In the deepest necrotic portion he found other bacteria. The best pictures were obtained when Weigert's modification of Gram's stain was used. If Gram's method was used care must be made in differentiation. The spirilla were stained best by carbol-fuchsin used for 24 hours. The author did not believe that the bacillus was a part of the mycelium-forming organism.

Rona in 1905 reported three cases of noma, two following measles. In all of the cases fusiform bacilli appeared to play an important rôle. In one case "spirochaete" were associated with the bacilli, the two organisms being found in pure culture at the border of the necrotic tissue. In one case a few "streptothricen" were seen in the necrotic tissue. In the deep tissue only the bacilli were present and they were in enormous numbers.

Buday in 1905 found in two cases of noma many different kinds of bacteria in the necrotic tissue. On the surface were cocci, diphtheria-like bacilli, colon bacilli, "spirilla," long threads, and fusiform bacilli. In the deeper parts filaments were seen in enormous numbers. Nearer the border of the necrosis thick groups of fusiform bacilli were seen. Here comma-shaped bacilli were also observed. At the border of the necrotic tissue and in the living tissue spirilla were observed in large numbers. He does not consider the fusiform bacillus and spirillum to be different forms of one organism. He found carbol-fuchsin best for staining the spirochaetae, using it for from six to 24 hours. The fusiform bacilli held Gram's stain quite long. The bacilli stained by Weigert's modification of Gram's method. He concludes that mouth and pharyngeal gangrene are due to different kinds of mouth bacteria, especially the symbiosis of a spirillum and a fusiform bacillus.

Pollard in 1905 found fusiform bacilli and "spirochaetae" in three cases of nosocomial gangrene in ulcers on the leg. He considers the fusiform bacilli the exciting agent and the spirochaetae simply saprophytes.

Herrman believes that the organisms called by him "spirochaetae" of necrosis, corresponding to the streptothrix of Seiffert and Perthes play the most important part in the etiology of noma. He considers that this organism is identical with that found by Plaut, Bernheim, Vincent, and others in ulceromembranous lesion of the mouth and with the *Spirillum sputigenum* and the *Spirochaete dentium* of Miller found normally in the mouth. He believes that they are different stages in the development of the same organism, and that this organism is not a bacillus but belongs to the family of spirochaetae.

Herrman calls attention to the fact that gangrenous processes similar to noma have been observed in lower animals. According to Roux the *Bacillus necrophorus* (Flügge) is identical with the *Bacillus diphtheriae vibulorum* (Loeffler), necrose bacillus (Bang), *Bacillus necroseos* (Salmonsen), *Streptothrix cuniculi* (Gasperini), *Actinomyces necrophorus* (Nuekirsch), and *Streptothrix necrophora*. Jensen calls this organism the *Bacillus necroseos* or the bacillus of necrosis.

According to Herrman this organism occurs in necrotic processes in the horse, cow, pig, kangaroo, ape, stag, antelope, and rabbit. Guinea-pigs, cats, and pigeons appear to be immune. This author describes the organisms as occurring in the form

of slender, straight, or curved rods. Several may be joined end-to-end, forming threadlike structures, and finer filaments are also seen. Schmorl also observed micrococcus-like bodies which, according to Herrman, may represent a stage in the development of new organisms. The organisms stain moderately well with carbol-fuchsin and carbol-thionine. Some show interrupted staining, some deeply stained, round bodies. The organism is an anaerobe, growing best in serum and blood-serum agar. The organisms are found penetrating apparently healthy tissue beneath the necrotic area in which they do not thrive.

From the similarity in the clinical, bacteriologic, and histologic characteristics Herrman thinks it probable that these necrotic processes in animals are analogous to noma in human beings, and that the organisms present in both are closely related.

Several of the observers just cited obtained a variety of bacteria, including cocci (staphylococci and streptococci) and pseudodiphtheria bacilli, from the tissues which had been dead for some time. Whether all these authors really described the same organism is doubtful. The various statements regarding the reaction exhibited to Gram's and Wiegert's stain are confusing. The fine threads described by Seiffert and Perthes and some of the later writers stain very faintly by Gram's method and are decolorized if care is not taken to avoid it. In this connection it must be remembered that, for purposes of differentiation, little dependence can be placed upon statements as to the staining or non-staining of a bacterium by Gram's method. The final result will vary with the thickness of the preparation, the composition of the staining fluid, the time the stain is allowed to act, and the degree to which the decolorization is carried. Partial decolorization becomes complete decolorization if the process is prolonged a little. The danger of depending upon Gram's stain for the differentiation of bacteria in sections of tissues is greater than in fresh smear preparations, because of alterations in the staining properties produced by various fixing and hardening agents.

That the delicate threadlike organism of Seiffert has sometimes been overlooked or mistaken for delicate connective tissue remnants is most probable, because of the faintness and difficulty with which it is stained. Bartels was at first in doubt whether the threads in his sections were not elastic fibers, but demonstrated that they were not by the absence of characteristic staining reaction.

From the study of the case of facial noma herein reported, the authors believe the organisms observed in the sections at the line of advancing necrosis are the threaded and spiral forms of one organism. The forms observed in the tissues correspond with the forms shown by us to occur in pure cultures of fusiform bacilli. These organisms have been described in previous articles (see Bibliography).*

The other organism which has been observed in connection with noma is the diphtheria bacillus. The first mention of the presence of the diphtheria bacillus in connection with noma was made by Bishop and Ryan in 1895. In the case reported by them, the bacteriologic examination was made by one of us (Weaver), and a diphtheria bacillus with little virulence for guinea-pigs was isolated in pure culture from the deepest part of the diseased tissue. No anaerobic cultures were made. No delicate threads were observed, but may have been present and overlooked.

* One of us (Tunnicliff) has been criticized by Mühlens (*Deutsch. med. Wchnschr.*, 1906, 20, p. 797), for not distinguishing between "Spirilla" and "Spirochaetae." At this time knowledge of these organisms is too limited to warrant an attack upon the use of terms which have both been applied by various authors to apparently the same organisms.

In 1896 Nicolaysen described in two cases a polymorphous bacillus, staining by Gram's method, and resembling the diphtheria bacillus, but possessing no pathogenic properties.

Saft, in 1898, cultivated from one case a bacillus which he describes as diphtheria-like.

Petruschky and Freymuth in 1898 reported a case of genital noma in a girl of three years, following measles. Smears from the necrotic surface contained various kinds of bacteria, among them some which resembled diphtheria bacilli. Two injections of antitoxin were given, 2,500 units in all. Recovery began soon after the second injection. By means of cultures diphtheria bacilli which were not highly virulent for guinea-pigs were isolated. Microscopic examination of the necrotic tissue gave a relatively limited number of typical diphtheria bacilli, and a large number of vibriones, bacilli, and fine spirilla.

Later in the year they reported a case of moderate gangrenous stomatitis in a boy of eight years following typhoid fever, from which a diphtheria bacillus was cultivated which was slightly pathogenic for guinea-pigs. Microscopically abundant spirilla and bent bacilli were found, corresponding to those of Bernheim, Miller, and Abel. The child recovered after receiving diphtheria antitoxin.

Passini and Leiner in 1899 reported a case of extensive noma in an eight-year-old tuberculous child, coming on without any acute disease. Preparations from the superficial parts contained long bacilli with pointed ends, resembling those described by Bernheim and others in stomatitis. In the deeper parts of the gangrenous tissue and at the border of the sound tissue, in smears and cultures they found diphtheria bacilli in pure culture. The diphtheria bacilli were pathogenic for guinea-pig and diphtheria antitoxin protected the animal against them.

Walsh in 1901 reported a series of eight cases of noma, occurring in St. Vincent's Home in Philadelphia, most of them following measles. From all of them he cultivated true diphtheria bacilli, which were shown to be virulent for animals. He considers the diphtheria bacillus as only one cause for noma.

Sailer in 1902 reported two cases of typhoid fever complicated by noma, from both of which diphtheria bacilli were cultivated. Guinea-pigs into which the cultures were injected were made sick for a few days, but recovered.

Other observers have found diphtheria bacilli associated with noma without concluding that they were the cause of the gangrene. Among such are Guizzeth, Hofmann and Küster, and Verhoeff.

In view of more recent studies of the pseudodiphtheria bacilli, it is not unlikely that some of the bacteria reported as diphtheria bacilli were really pseudodiphtheria bacilli. This is more probable as in most instances the bacteria were said to possess very slight virulence. In this connection it may be noted that E. H. Ruediger has found pseudodiphtheria bacilli associated with gangrenous anginas of scarlatina which are virulent for guinea-pigs.

The two varieties of bacteria already discussed are the only ones which have been found by any numbers of observers. Other bacteria have been encountered by a single or a few observers.

Froriep, *Chirurg. Kupfertafeln*, 1844, Taf. 438, 39, appears to have been the first author who attempted to demonstrate a causal relationship between vegetable organisms and noma. In sections from a rapidly fatal case he pictured small, round structures lying between muscle fibers and connective-tissue cells.

In 1872, Strueh studied the necrotic tissues from a case in an adult and found what he believed to be the same organisms as pictured by Froriep, which he described as rather thick and double-contoured. They were associated with leptothrix and he designates them as "nomapilz."

Babes and Zambilovici in 1892–93 found in two cases of noma a slender aerobic liquefying bacillus, which grew well on most media and did not stain by Gram's method. It caused gangrene in the cheeks of rabbits. They considered it the cause of the gangrene, although they also described abundant, undulating, and ramifying filaments resembling actinomyces in the tissue. Guizzetti in 1896 found the same bacillus in one case, which produced gangrene and abscesses in experimental animals.

Schimmelbusch in 1889 described an aerobic bacillus which he cultivated from a single case. It grew readily on all kinds of media, and produced necrosis and abscesses in experimental animals.

Longo in 1903 cultivated from a case of noma an aerobic bacillus, which corresponded in most respects to the one described by Babes and Zambilovici.

Hofmann and Küster in 1904 isolated a sporulating aerobic bacillus from a case of noma which they believed to be etiologically connected with the disease.

Some writers have been inclined to consider the streptococcus the most important factor in the causation of noma, among them being Ranke, Holt, and Verhoeff.

Almost everyone who has made bacteriologic studies of noma, has found a great variety of organisms in the older gangrenous tissues. Because of this and because of the lack of uniformity in results, many have concluded that noma is due to the combined action of bacteria from the mouth acting upon specially vulnerable tissues. In this group are Baumgarten, Comba, Durante, Trambusti, and Strada. Rosenbach compares this infection to that of tetanus, malignant edema, and rauschbrand, in which saprophytes under favorable conditions invade the tissues.

The organisms observed in the nomatous tissues at the line of advancing necrosis appear to resemble very closely certain bacteria found in the mouth under diseased and normal conditions. Attention has been called to this by Herrman, Rona, Buday, and others. Further study must determine what relation the bacteria found in the tissues bear to the necrosis, and in what they differ from normal oral bacteria if they really do so differ. According to Rona, 1905, gangrenous, diphtheritic phagedenic chancre is a local infection similar to gangrene in which "spirilla" and bacilli are present on the surface and the bacilli alone in the deeper tissues. He considers that Vincent's angina, ulcerous stomatitis, gangrenous stomatitis, and noma must be considered identical, differing only in degree, on account of the similarity of their location, clinical course and pathological anatomy, their character and histological structure, and the morphological and staining properties of the bacteria. He thinks that the spirilla and bacilli cannot be differentiated from Vincent's organisms found in hospital gangrene. The same organisms were found by him in pulmonary gangrene. He believes that ulcerous stomatitis and mercurial gangrene differ from each other only in that in the latter mercury causes the lower resistance. He considers Miller's organisms found in the mouth to be the same as the ones found in all these various conditions. He says that the clinical, anatomical, and bacteriological similarity make one think of a common etiology, but that this must be determined by cultures and animal experiments.

Buday, from the study of cases of noma, gangrenous pharyngitis, gingivitis, and stomatitis, believes them to be different types of the same process. He found the patho-

logical anatomy and bacteriology to be the same. He considers that these lesions are due to different kinds of mouth bacteria, especially the symbiosis of a spirillum and fusiform bacillus.

Rona calls attention to the fact that noma of the face begins without exception in gangrenous stomatitis. If the fusiform bacilli or spirilla found in the mouth are etiological factors in the gangrenous stomatitis, as these organisms are found in such abundance in the tissue of noma cases, it would seem probable that the organisms are the same. Since in all of these conditions, lowered resistance plays such an important rôle, he believes that under such conditions these saprophytic mouth bacteria may become virulent.

PATHOLOGICAL ANATOMY.

The gangrene is most often observed to involve the tissues about the mouth and those about the genito-anal region. In girls the genital gangrene comprises quite a large percentage of the cases. The conditions which favor the peculiar form of infection seem to be furnished by both locations. The primary gangrene may also involve the tissues of the external ear. The gangrene may be limited to any one of these locations, or may appear simultaneously or consecutively in two or more.

Of 16 cases observed by Blumer and MacFarlane, the mouth was alone involved in four; the mouth and other parts, i. e., ear and vulva, in three; the vulva alone in two. In three cases the rectum alone was affected and in five the rectum was involved together with other parts. The frequent association of gangrene of the face with that of the external genitals has been remarked by Blumer and MacFarlane, Bouchut, Orth, and Richter. Gangrene of the external genitalia alone has been noted by Blumer and MacFarlane, Gierke, Mayr; and Wood.

Among those speaking of gangrene of the rectum alone or combined with a similar lesion elsewhere, are Blumer and MacFarlane and Bouchut. Gangrene of the tissues in the region of the external ear have been spoken of by Blumer and MacFarlane, Gierke, Holt, and Mayr.

According to Tourdes, Taupin observed gangrene of the lungs, pharynx, esophagus, and stomach, in eight out of 36 cases. Barthez and Rilliet, out of 20 cases found gangrene of the lungs and pharynx four times.

Tourdes says that the gangrene occupies most often the middle of the cheek, next in frequency the lips, especially the lower. It can destroy half the face, the neck, eyelids, forehead, and the nose. Less often it attacks the arch of the palate, especially the posterior part, the uvula, the tongue, and the floor of the mouth. It is unusual for both sides of the face to be affected at the same time. Some statistics seem to show that the left side is more often involved than the right.

In the following description as in other places the excellent work by Barthez and Rilliet, based upon examination of 20 cases, has been largely utilized.

Skin.—In fatal cases of noma there is more or less involvement of the skin which lies over the deeper tissues affected. The cheek or lip is swollen, violet or greenish, tense and shining as if oiled. Over the highest part of the swelling there is often seen a circular or oval, regularly outlined, gangrenous eschar, varying from 1 cm. to 3 cm. or so in diameter. In other cases the sequestrum is larger, involving irregularly varying parts of the face, and extending to the chin, neck, nose, eyelid, and even as far as the ear. Half or even almost the whole face may be involved. The eschar is always black, and usually is dry like parchment. It may extend only 1 to 2 mm.

into the skin, or entirely through it, and rarely ever involves the underlying tissues. After the sequestrum has separated there remains a perforation of the soft parts, revealing the deeper parts and even the interior of the buccal cavity.

Mucous membrane.—When the cutaneous surface has undergone gangrene, there is always involvement of the mucous membrane. It may, as in the skin, be limited and appear as an ulcer with a grayish-black base, lying in the fold between the cheek and gum, or upon the cheek opposite the space between the rows of teeth. On the other hand, the destruction of the mucous membrane may be extensive, extending from the angle of the mouth back to the soft palate. There is destruction of the mucous membrane through its entire thickness, the surface presenting a black or brown semi-fluid mass which is readily scraped away and contains structureless fragments of the necrotic mucous membrane. There is usually partial or complete destruction of the gums on the corresponding side.

Jaw-bones and teeth.—With gangrene of the gums the teeth are loose and easily extracted or have already fallen out. Incisors, canines, or molars may be affected. With the destruction of the periosteum the jaw becomes denuded and necrotic, and fragments may be loosened.

Tissues between the skin and mucous membrane.—In the simplest condition the fatty tissues and muscles of the cheek are infiltrated with serum, but still retain their structure and form a firm mass deep in the cheek. If the process is more severe or advanced, gangrenous changes are also observed in the deeper structures, especially where they approach the affected skin and mucous membrane, and thus a brownish putrifying mass, 5–8 mm. thick, is seen, beyond which the fatty tissues and muscles are infiltrated with serum, appearing homogeneous and structureless. Rarely the entire thickness of the cheek or lip appears gangrenous, and if the slough has separated a perforation is observed.

Vessels and nerves.—In the tissues which are only infiltrated the vessels are permeable and their walls scarcely at all thickened. At the border of the gangrenous tissue the vessel walls are thickened and begin to have the appearance of gangrenous tissues. In the interior of the gangrenous tissues the vessels can still be followed, but their lumina are closed by coagula. The coagulum stops abruptly at the limit of the gangrenous tissues. Sometimes a gangrenous mass is found within an artery. The veins may contain an ichorous material. Nerves were examined once and in the middle of the gangrenous area they had the external appearance of the surrounding tissues. On section the "Nerven-mark" appeared normal. The ductus of Stenson was examined once and was found permeable throughout, and opened freely in the center of a gangrenous remnant of mucous membrane.

In gangrene of the vulva, the lesions are similar to those observed in the cases in which the face is involved. Tissue destruction may be extensive and extend to the structures about the rectum.

MICROSCOPIC ANATOMY.

The microscopic appearances presented by nomatous tissues have been described more or less in detail by a number of authors during the past 15 years. The following is based upon the descriptions of Bartels, Bishop and Ryan, Blumer and MacFarlane, Foote, Krahn, Kraus, LeCount, Matzenauer, Perthes, Schimmelbusch Schmidt, and Verhoeff. All these descriptions accord in the main. Schimmelbusch characterized noma as an acute mortification of tissue without reaction of neigh-

boring tissues worthy of notice. A sharp line of demarkation between the living and necrotic tissues is mentioned by most writers. Several authors speak of three distinct zones: (1) necrotic; (2) middle zone of infiltration with round cells; (3) normal tissues. The degree of infiltration in the middle zone has presented great variation in different cases, from the extreme cases of Verhoeff in which there was no inflammatory reaction, to those in which infiltration is described as extensive. Others say that the small cell infiltration is slight. In general it would seem that the degree of infiltration is largely dependent upon the rapidity with which the gangrene is advancing at the point examined, the accumulation being absent or very limited when the gangrene is progressing rapidly, and being abundant when the necrosis is at a standstill or making little headway. In the transitional zone, between the tissues which are necrotic and those which are the seat of inflammatory reaction, Matzenauer describes a wall consisting of a network of fibrin. Fibrin masses are often found deep in the tissues about the vessels which are dilated and distended with blood. The walls of the vessels in the inflammatory zone early present the appearance of coagulation necrosis, and, in preparations stained by Weigert's method, are surrounded by an intensely staining network of fibrin. Schmidt says that near the boundary zone there is marked intermuscular edema, the muscle fibers presenting a swollen appearance. The nuclei of connective tissue and muscle cells are the first to exhibit degenerative changes. The mucous glands appear most resistant.

The zone of necrotic tissue takes a diffuse stain, the structural outlines of muscle, glands, and other structures being often preserved, but all are lacking in nuclei which stain. There are also altered blood cells and fibrin. In the necrotic zone are seen many strands of elastic connective tissue which seem to be most resistant to the necrotic process. Bartels described mast-cells in the zones of necrosis and inflammatory reaction. He also says that in one case the nerve fibers in the necrotic parts were almost unaltered.

SYMPTOMS.

According to Tourdes and other observers there are usually no constitutional symptoms until the gangrene is well established. After the disease has existed for several days symptoms of marked prostration and sepsis develop, sometimes quite rapidly (Holt). As a rule the patient is suffering or convalescing from some severe disease so that the symptoms are masked by those accompanying the primary trouble.

Face.—It is usually pale at the onset and remains so during the entire course. In the cases observed by Barthez and Rilliet, the face became somewhat red during the last day of the illness in one case, and yellowish in another. The parts unaffected by the gangrene are often marmolated violet. There is often an edema of the eyelids, but if it is absent the eyes are sunken. The lips are covered with crusts. The alae nasi are usually dilated. The nose is pointed, when not involved in the gangrene. The parts affected loose all expression.

General expression.—The patient is usually quiet and woeful, often languid and peevish, sometimes very fretful and ill-natured. Rarely the child remains in good spirits. The appearance is often that of a cachectic or acute secondary disease.

Strength and position.—Baron always found prostration of strength; in the case of Destries the strength was maintained throughout the course of the disease. Some children are completely prostrated, others suffer little from loss of strength and sit up in bed, interested in their surroundings to the last day. One case of Barthez and

Rilliet got out of bed unassisted the day before death. Destries' child played cards when the gangrene was at its height. The children showing great prostration have usually been greatly weakened by some preceding disease.

Pulse.—It is usually said to be frequent and very small. The character depends largely upon the accompanying conditions. In some cases in which gangrene was the chief disease, Barthez and Rilliet noted that at the onset there was slight increase in the frequency of the pulse, being 80 to 100 or 120, and that it never exceeded this even in very young children, but always toward the end became small and imperceptible. In cases appearing in the course of other acute or chronic diseases the pulse was observed to become more rapid soon after the gangrene began, reaching 120 to 140.

Fever.—According to Tourdes there is no fever as a rule, even in very young children, unless there are complications. In some the skin is hot and feverish, in others cool or cold. The difference depends upon the accompanying disease. Gierke found the fever to be variable. According to Holt the temperature is usually elevated to 102°–103° F., sometimes rising to 104°–105° F. He says it may become subnormal before death.

Sweats.—The skin is always dry rather than moist. According to Tourdes viscid perspiration has been observed, probably an agonal phenomenon.

Anasarca is rare. Bartels found in a woman 40 years of age general edema as death approached.

Digestive system.—Children usually retain their appetite and even desire to eat to the last moment. In a case of Barthez and Rilliet, the appetite had been partly lost from another disease, but improved with the onset and during the course of the gangrene. Thirst is usually marked, and patients drink greedily. The tongue is always moist, sometimes yellowish, rarely red. In some instances it becomes black on the affected side from involvement in the gangrene. In the cases observed by Barthez and Rilliet, vomiting was never observed, but diarrhea was always present. In a case of a woman 40 years of age, observed by Bartels, there was diarrhea toward the end. Gierke usually but not always found diarrhea present. It occurs when the gangrenous tissue breaks down, and is probably due to swallowing fragments of the tissue. Of the eight cases of noma of the genitalia and external ear observed by Gierke, slight diarrhea of short duration occurred in only one.

Respiratory system.—Frequently pneumonia, most often broncho-pneumonia, occurs as a complication.

Nervous system.—Barthez and Rilliet never observed nervous symptoms. In the case of Destries and in several of those of Baron, sleeplessness and delirium were present. Gierke says that a few days before death all children become restless, later apathetic, and die in a state of exhaustion. Delirium was present in none of Gierke's cases.

Renal symptoms.—Bartels reports slight albuminuria in a woman 40 years old.

PHYSICAL SIGNS.

I. FACIAL GANGRENE.

The mucous membrane, the deep tissues, and the skin have been considered as sites for the first appearance of this disease.

The gangrene almost always begins in the mucous membrane. This was found

to be true in all cases observed at the beginning by Barthez and Rilliet, and their observations accord with those of Destriel, Baron, Tourdes, Bouley, and Caillault. Bartels reports a case in a child three years old in which the necrosis began on the left tonsil and extended to the palate and pharynx. Bouchut believes that aphtha, which has become gangrenous, is always the source of the gangrene which involves the cheek. Brüning reports four cases of which two developed from an ulcerative stomatitis and two seemed to begin in an ulceration of the hard palate. Coates thinks that the disease generally starts at the edge of the gums. He never saw a case begin on the inside of the cheek. Filatov considers that noma generally develops from "stomacace." Henoch believes that it almost always begins in ulcerative stomatitis. Mayr in 14 cases after measles observed it to develop seven times from "stomacace" and "nymphacace," once from an excised "abscesse excarié," five times from gangrenous vesicles upon the inner side of the cheek, and once from an eczematous inflammation of the external ear. Bohn observed the gangrene to develop from a stomatitis. According to Allbutt it occasionally follows ordinary ulcerative stomatitis.

A. L. Richter, Delaberge, and Mouneret and Billard all agree that the first sign of the disease is often a hard, indolent swelling of the size of an almond, lying deeply in the tissues and accompanied by some redness. Taupin, Gierke, and Henoch also believe that gangrene may begin in the deep tissues of the cheek. Tourdes thinks if gangrene ever does originate here, it is exceptional. Barthez and Rilliet know of but one well-authenticated case, that of Key.

Weber, quoted by Tourdes, observed a case in which the gangrene began in the skin. Bohn (1880) says that there are no authentic cases in which noma originated in the cutaneous surface of the cheek. Kraus (1902) quotes Bainbridge as finding two cases out of 100 which developed from without. Filatov (1904) says "noma sometimes begins with the skin of the cheek."

Barthez and Rilliet from their own experience and that of others conclude that gangrene may begin in three ways:

1. Most often with ulceration followed by gangrene of the mucous membrane.
2. With an edematous swelling followed by gangrene of the mucous membrane.
3. Gangrene of the deeper parts, extending later to the mucous membrane and skin.

Mucous membrane.—Baron and Destries always observed upon the mucous membrane first a small aphtha or phyctena, which later enlarged. Barthez and Rilliet observed on the first day an ulcer upon the mucous membrane similar to that of stomatitis, either on the alveolar border of the gums, in the gingivo-buccal fold, or on the central part of the cheek opposite the space between the rows of teeth. This ulcer was sometimes very small, a few millimeters in extent, with a gray or a distinctly gangrenous covering.

Bouley and Caillault claimed the ulcer was always gangrenous from the first day, but this is certainly not in accord with the observations of other writers. Sometimes the ulcer remains a long time and forms a true stomatitis which passes through its phases and terminates with gangrene. This has been observed by Barthez and Rilliet.

When gangrene has once begun, the ulcers spread quite rapidly; they bleed readily, become gray, then black and covered with a fluid ichor. The extension of the gangrenous process is more or less rapid, and in from three to 16 days may involve the entire mucous membrane of the affected side. Later the gangrenous parts become

detached in fragments, and fall into the mouth, or are pulled off by the child without pain.

Teeth and jaw-bones.—With the gangrenous destruction of the gums the teeth become loosened and fall out. The jaw, denuded of its periosteum, becomes necrotic to a corresponding extent. If life is sufficiently long preserved, or if recovery takes place, necrotic fragments of jaw may separate and be discharged. Such fragments may carry teeth with them. The necrosis may extend beyond the jaw-bone to the malar, temporal, and orbital bones.

Salivation.—There is usually a profuse flow of saliva, which early is sanguinated, and soon becomes black or brown with the gangrenous fragments in suspension.

Odor.—The odor from the mouth is very offensive and characteristic. The breath is usually fetid from the first few days, sometimes even before one notices a change in the buccal mucous membrane. It is often the first thing to excite attention. Of itself the odor is not enough upon which to base a diagnosis, as pseudo-membranous stomatitis may emit a very similar odor. Henoch says that the odor is not only fetid, but gangrenous, and may not be detected readily.

Skin and underlying tissues.—There may be an edematous swelling of the lips and cheek before the gangrene appears, which is like that observed in ulcerative stomatitis. This is usually present one or two days before the hard central swelling appears.

Swelling of the submaxillary glands.—Taupin, quoted by Tourdes, observed an enlargement of the submaxillary glands in four cases out of 36. Boeckel and Constant also encountered it (Tourdes). Most of the earlier physicians have not mentioned any glandular swelling. Barthez and Rilliet once found them enlarged to the size of pigeon's eggs, grayish-red on section, soft, and filled with some fluid, but not suppurating. Bartels observed enlargement of the submaxillary glands in a woman of 40 and a child of three years. According to Bruns the lymph glands are not usually enlarged. Carrière-Montjosieu says the glands are not enlarged unless there is an added microbic infection. An enlargement of the submaxillary glands has been observed by later writers, as Henoch and Filatov.

Pain.—Noma is generally described as a painless affection (Allbutt, Blumer and MacFarlane, Filatov, Gierke, Tourdes). According to Holt the pain is rarely severe; in many cases it is absent. Carrière-Montjosieu reports a case of noma in which pain was intense.

Usually about the first to the third day of the gangrene there forms deep in the tissues a firm, hard, circumscribed kernel, 1–2 cm. in diameter. The overlying skin is then tense, shining as if oiled, often violet or marmolated. The hard kernel may not form until later, even on the sixth to the ninth day, and shortly before death. It was never absent in the cases observed by Barthez and Rilliet. Later it may soften because of gangrenous disintegration of the tissues entering into its formation. According to Filatov: "In the beginning of the disease there always appears a tumor in the cheek, the integument being pale, not hot, and painless on pressure."

In many cases an eschar forms on the cheek. According to Baron it occurs on the second or third day. In Destries' cases it occurred on the sixth day. Out of 21 cases observed by Barthez and Rilliet, it appeared between the third and seventh days in eight, on the 12th day in one, and on the 17th day in one. At the place where the eschar is to form the skin becomes violet and then black. Often a vesicle forms at the summit of the swelling of the cheek or lip, and soon it becomes

included in a gangrenous spot. The gangrene of the skin tends to extend quite rapidly, and is surrounded by edematous tissues. Barthez and Rilliet describe two distinct zones surrounding the circular eschar which were first pointed out by Bouley and Caillault. The first zone is from 5 to 6 mm. wide, circumscribes the gangrenous eschar, is gray, and bleeds freely. The second zone is formed of a diffuse edema which spreads more or less widely. The skin at the border of the edema is usually pale, while that near the gray zone is at times moderately or quite red.

If the eschar becomes circumscribed it separates at the edges, and finally falls away with all the underlying tissues down to the mucous membrane, so that a perforation results, through which flows saliva charged with gangrenous masses from the mouth, and exposing to view the interior of the mouth and nose. The edge of the perforation remains swollen, hard and red, or marked by gangrenous shreds.

If healing occurs the borders become clean and finally assume the appearance of a granulating surface. As the edges approach a fistula remains, which may be permanent or may completely close.

II. VULVAR AND AURAL GANGRENE.

According to Kraus, vulvar and aural gangrene have a course similar to buccal, but multiple areas are more common. Vulvar gangrene usually originates in an ulceration of the mucous membrane of the labia, but may begin deep in the tissues (Henoch). Holt, who has observed noma of the ear seven times, found it always preceded by chronic otitis media. It began in the deeper structures of the canal, the first symptom noticed being a nodular swelling, just beneath the ear, crowding the lobe upward. Shortly afterward there appeared a dirty brown discharge with a gangrenous odor. The gangrene extended gradually, until in some cases the whole side of the face and head was involved.

COURSE, DURATION, AND TERMINATION.

Course.—According to Tourdes there are three periods in the course of noma:

1. The period of ulceration of the mucous membrane, edema of the face, and the formation of the central kernel, lasting two to three days.
2. The period of gangrene, extending from five to 12 days.
3. The period of general infection.

Duration.—Noma has a rapid course rarely extending over more than two weeks. Death usually occurs between the eighth and 14th days.

Relapses.—There are few records of relapses on account of the disease being so fatal (Barthez and Rilliet). Berthe (1754), quoted by Tourdes, saw a child attacked by noma twice. Hueter reported a case having a relapse followed by recovery. Ziegler observed two relapses in a case. According to Gierke in seven cases, beginning healing appeared and death followed from pneumonia. In one case observed by him a relapse occurred, and the child recovered a second time in six months. In another case of Gierke's the second attack, occurring two years after the first, proved fatal.

DIAGNOSIS.

Noma is easily recognized. The diagnosis is based on the gangrenous odor of the mouth, the flow of ichorous and fetid saliva, the grayish ulceration of the mucosa, the central kernel, the yellowish or brownish spot on the skin, the rapid course, and destruction of tissue.

Taupin considered (1839) noma and pseudo-membranous or ulcerative stomatitis the same in general causation, and nature. This view is held generally by modern observers who have studied the bacteriology of the affections (Buday, Rona, and others).

Malignant pustule always begins in the outside skin, which rarely if ever is the case with noma.

Gangrenous aphthae is a disease of the same nature, but there is only a localized eschar, while in noma gangrene is diffuse.

COMPLICATIONS.

As noma always develops after some other disease it is difficult to specify the complications.

The most frequent one is pneumonia. It was present in 58 out of 63 cases collected by Tourdes. It was absent in only two out of 21 cases examined by Barthez and Rilliet.

Pleurisy and enteritis have been observed to develop in the course of gangrene; but the complications most grave are gangrene in other parts of the body (Tourdes). In eight out of 36 cases reported by Taupin, gangrene was present in the lung, pharynx, esophagus, and stomach. Barthez and Rilliet found gangrene of the lungs and pharynx in four out of 20 cases. Coincident gangrene of the vulva has been observed by Richter and others.

Hemorrhage rarely occurs. Hueter saw a fatal case. There was a severe nasal hemorrhage in the case observed by us.

PROGNOSIS.

All observers agree that noma is usually fatal. According to Tourdes the 30 cases seen by Baron were all fatal, as well as the 36 cases of Taupin. Tourdes collected 239 cases from the literature, of which 176 died, making a mortality of 73 per cent.

According to Tourdes the factors which influence mortality are age, both extreme youth and old age being unfavorable; bad hygienic surroundings; complications, especially pneumonia; the nature and time of treatment. The extent of destruction of tissue is not always the cause of death.

Cases in which recovery occurs almost always are uncomplicated by any severe affection and those in which the appetite and strength are preserved throughout the entire course. If recovery does occur it requires weeks and even months for healing according to Gierke. If the part of the jaw containing rudimentary teeth has been destroyed, the mouth will remain toothless. Speech is always affected. Generally a great deformity results. In healing after gangrene of the vulva, fistulae, stenosis, or atresia of the vagina may result (Holt). Nine out of 14 cases seen by Mayr died. The five saved were those with least extension of the disease and best body resistance.

Bouchut says that gangrene of the mouth which involves a large part of the face is fatal. Gangrene of the mouth, according to him, heals only at its onset under the influence of deep cauterization, which is repeated two or three times daily with hydrochloric acid.

Gierke observed 18 deaths and two recoveries. No cases of his died after the second week. Some cases of circumscribed noma without deeply extending involve-

ment ended fatally. All of the cases of noma of the genitalia and auditory region observed by him were fatal.

Springer (1904) gives a table of all cases of noma in the Children's Hospital in Prague, between 1888 and 1903, during which time they were treated by means of excision. Twenty-three cases were observed, two recovered, giving a mortality of 90.5 per cent. Of the 16 cases operated upon 14 died, giving a mortality of 87.5 per cent. Of the seven cases not operated upon, seven died giving a mortality of 100 per cent.

TABLE 1.

SUMMARY-PROGNOSIS.

Author	Year	No. of Cases	Died	Recovered	Percentage
Wood	1816	12	10	2	83
Tourdes	1848	239	176	63	73
Mayr	1852	14	9	5	64
Barthez and Rilliet. Baron and Destries	1855	29	26	3	89
Bruns	1859	413	290	123	70
West	1866	10	8	2	80
Gierke	1868	18	16	2	88
Steiner*	1876	108	103	5	95
Day	1881	7	6	1	85
Woronichin	1887	22	19	3	86
Blumer and MacFarlane	1901	16	14	2	87
Springer	1904	88	83	5	94
Total		976	760	216	77.8

*Cited by Bohn.

TREATMENT.

The high mortality under the treatment employed by all observers serves to emphasize the fact that most of the measures which have been used are of little or no value. Stoppage of cases in the early stages has been reported as due to various local measures. It is in the early stage that something may be expected. The prompt removal of the tissues involved, together with some apparently healthy tissue, offers a reasonable hope of stopping the progress of the disease. Such measures have yielded good results in the hands of Ranke, Springer, and others. The knife is used first to remove the necrotic tissues and is followed by the free use of the actual cautery. Of course this is done under the anesthesia, and the likelihood of favorable results is in proportion to the thoroughness with which the operation is carried out.

Aside from free removal, local antiseptic measures are advisable, but can scarcely be looked upon as curative.

Patients recovered from noma should not be subjected to plastic operations for the repair of deformities for a number of months after complete recovery, because of the danger of the recurrence of gangrene in the operation wounds.

Aside from measures directed directly toward the local condition, every effort is to be made to support the strength of the child by suitable food and tonic measures.

Of more importance than to cure is to prevent the occurrence of noma. This must be accomplished by placing children who are liable to the disease in the best hygienic conditions where they will be freely supplied with good food, fresh air, and sunshine. The mouths and genitalia of delicate children who have been reduced by infectious diseases should be carefully watched. Any ulcerative lesion of the mucous membrane is to be vigorously cared for until healed. If any gangrene appears,

radical measures should be carried out at once. Cases of noma should be isolated, since the danger of contagion is always present.

AUTHORS' CASE.

The following case was observed in the Hospital of the Memorial Institute for Infectious Diseases in the service of Dr. Frank Billings and Dr. Alexander F. Stevenson, to whom we wish to express our thanks for the opportunity of reporting the case.

Clinical record.—O. A., female; eight years. The patient's family history is negative. She has had none of the diseases of childhood. A few weeks previous to her entrance she suffered from an alveolar abscess which caused considerable swelling of the left side of her face. On September 21, 1904, the patient was taken ill with sore throat, vomiting, diarrhea, and fever. The following day a light red rash appeared and a diagnosis of scarlet fever was made. She was admitted on September 24.

She is a poorly nourished child. There is a slight conjunctivitis of both eyes. She has considerable muco-purulent discharge from the nose. Her lips are bleeding and covered with crusts. Her teeth are badly decayed and covered with tartar. Both tonsils are much swollen, of a brilliant red color, and are free from exudate and membrane. The mucosa of the whole mouth is of a bright scarlet color. Her tongue is free from coating, very red, and the papillae stand out prominently. There is a large amount of grayish mucus in the throat.

Her whole body with the exception of the area around the mouth is covered with a brilliant red rash, disappearing readily on pressure. It is punctate in character, except on the hands and feet, where it is uniform. Both drum membranes are pinker than normal.

The posterior cervical, axillary, and inguinal glands are the size of peas. The anterior cervical glands are the size of beans individually, and altogether form a mass the size of a walnut.

Heart and lungs are normal. Temperature at entrance, 102° F.; pulse, 140; respiration, 28. Stools are green and contain mucus.

Smears from the tonsils show leucocytes filled with streptococci and a few diplococci. Both organisms are also found outside of the cells. Cultures on blood serum from the tonsils show streptococci and staphylococci and a few bacilli. Blood cultures are sterile. Cultures from the nose show staphylococci.

September 26.—A thin white, exudate has formed on both tonsils. It is easily removed without leaving a bleeding surface. Smears and cultures show streptococci and diplococci. Catheterized specimen of urine shows a trace of albumin and a few granular casts. She is delirious at night.

September 27.—A membrane has formed over both tonsils and the anterior pillars of the fauces. It is thick, soft, and of a buff color. Smears show the same organisms as before.

September 28.—The left cervical glands are enlarged, being the size of two walnuts.

September 29.—Desquamation has commensed. Smears from the membrane on the tonsils show streptococci, spirilla, and diplococci.

October 2.—There is a moderate amount of yellowish purulent discharge from the left ear.

October 3.—There is a purulent discharge from the right ear. Cultures from the ears show streptococci and staphylococci. The tonsils appear less swollen and

red. The membrane is present only on the anterior pillars. There is no discharge from the nose. There is paralysis of the pharynx, fluids drunk running out of the left nostril.

October 4.—The left side of the face is swollen and tender. The cervical glands on the left side extend from the mastoid process to the angle of the jaw. Cultures from the throat show streptococci and staphylococci. Pulse is weak. The tonsils appear almost normal in color, slightly swollen and free from membrane. There is more discharge from the left ear than from the right. The left side of the face is more swollen and slightly pink in color.

October 6.—There is an increase in the muco-purulent discharge from the left nostril. Smear preparations show many spirilla, fusiform bacilli and streptococci, diplococci and pseudodiphtheria bacilli. There is considerable swelling and tenderness of the superior maxilla of the left side. The upper and lower eyelids of the left eye are swollen, and there is some purulent discharge from it. Cultures from the discharge show staphylococci, pseudodiphtheria bacilli and streptococci. Blood cultures are sterile. Catheterized specimen of urine shows albumin, amorphous urates, epithelial cells, a few hyaline and granular casts, and leucocytes. Cultures from the urine show streptococci.

October 7.—Cultures from the nose give pseudodiphtheria bacilli, streptococci, and staphylococci. The swelling of the cheek and lip is greater and more tense. There is a large amount of muco-purulent discharge tinged with blood from the left side of the mouth, smears from which show spirilla, fusiform bacilli, streptococci, and pseudodiphtheria bacilli. The left side of the superior maxilla and the teeth on that side are covered with a yellowish green membrane. Her breath is very fetid. The upper four teeth beginning with the incisor on that side are loose.

October 8.—The eyelids of the right eye are swollen. The gums separate from the teeth on the left side, especially in front of the first tricuspid tooth, where a probe can be inserted for a distance of an inch, at which point the bone seems to be denuded. The membrane over the superior maxilla is partially separated and hangs into the mouth. At 4 P. M. the skin over the left molar bone is observed to be slightly blue in color.

October 9.—In the morning the discoloration on the upper inner side of the left cheek extends over an area 7 cm. in diameter. The color is blackish blue. It has a well-defined, bluish line of demarkation externally, reddish internally. The area is devoid of the outer layer of the skin. The eyelids of both eyes are edematous as well as the left side of the face extending behind the ear. The right side of the forehead is also edematous. The sloughing in the mouth extends almost to the median line. The first tricuspid teeth (upper and lower) have fallen out. The left side of the upper lip externally is black and there is a blue line of demarkation. The glands on the left side of the neck are less swollen. There seems to be some fluctuation in them. Fine and coarse rales may be heard over both sides of the chest. She coughs occasionally.

October 10.—The area of necrosis on the left side of the face is almost circular, being three and a half inches in diameter. It has extended over the lower eyelid almost to the middle of her nose and upper lip. It has extended into the left nostril and into the mouth, on the upper and to a slight extent on the lower lip. The sloughing has extended beyond the median line inside of the mouth, the first incisors on the

right side being loose. At 3 P. M. there is some hemorrhage from the right nostril. The patient died at 6 P. M.

From the throat fusiform bacilli and filaments were grown in mixed cultures in sugar-free broth and ascitic-fluid broth aerobically. Anaerobically, fusiform bacilli and filaments associated with other organisms grew in ascitic-fluid broth and sugar free broth.

The fusiform bacilli and filaments did not retain Gram's stain, except in the dark spots.

Neither fusiform bacilli nor filaments grew in the cultures from the necrotic tissue of the face, which may have been due to the small amount of material used for inoculation. In addition to streptococci, staphylococci and pseudodiphtheria bacilli were isolated from the necrotic tissue.

AUTOPSY (Dr. Ruediger).

Anatomical diagnosis.—Necrosis of left side of face involving the upper jaw. Acute nephritis. Hydropericardium. Double hydrothorax. Splenic infarcts. Acute splenic tumor. Suppuration of left submaxillary glands. Swelling of tonsils and of pharyngeal walls. Broncho-pneumonia. Desquamation of scarlet fever.

The body is that of a poorly nourished girl, 3 ft., 10 in. tall. Rigor mortis is present. There is marked postmortem discoloration on the back. On the feet, legs, hands, and wrists are signs of desquamation. On the left side of the face is a very dark-bluish, nearly circular area of necrosis extending from the median line of the nose to within 2 cm. from the ear, and from the upper margin of the lower eyelid to the lower jaw. The upper eyelid also has a bluish tinge. A zone about 1.5 cm. in width around the periphery of this nearly circular area is lighter colored and has a slightly greenish tinge. This area is soft and markedly edematous; the left eyelids are especially edematous. In the central and darkest portion of this area the epidermis is broken and a small amount of slightly hemorrhagic serum exudes. This area of necrosis involes also about three-fifths of the upper jaw and roof of the mouth on the left side, and the left three-fifths of the soft palate and uvula; also a small portion of the lower gums posteriorly. The upper teeth from the left molars to the right canine are missing.

The peritoneum is everywhere smooth and glistening. There are no adhesions. There is a small amount of slightly turbid, pale yellow fluid in the peritoneal cavity. The intestines are distended with gas. The omentum contains very little fat and reaches downward to a point about midway between the ensiform and the symphysis. The liver extends a little below the costal arch. The pleurae are smooth and glistening. There are no adhesions about either lung. The right pleura contains about 200 c.c. of hemorrhagic fluid, and the left pleura contains a somewhat smaller quantity of clear straw-colored fluid.

The pericardial sac is filled with a clear, straw-colored fluid. It also contains a gelatinous clot of fibrin about as large as a small walnut. The clot is not adherent. The pericardium and epicardium appear normal.

The thyroid shows no changes.

In the upper half of the larynx there is some slaty pigmentation. Otherwise there are no changes. The trachea is normal.

The peribronchial lymph glands are soft and bloody, and swollen.

The right lung has a grayish-pink, somewhat mottled appearance. There are

several slightly depressed areas which crepitate very feebly. These are dark red in color, and vary in size from 3 to 5 cm. The cut surface of the lung is hemorrhagic and has dark-red areas, corresponding to those seen on the surface, which are more bloody and solid than the surrounding tissue. Frothy blood can be expressed from the lung. The left lung is very much like the right, and the same description applies to it.

The heart is almost as large as the owner's fist. It is firm and apparently normal. The apex is formed by the left ventricle. The endocardium, myocardium, and all the valves are normal.

The aorta is normal.

The spleen is large, soft, and light red in color. On the surface are six firm, light-grayish-pink, irregular areas which are slightly depressed. The largest measures 3×1.8 cm., and is 1 cm. in depth on the surface where these areas are seen distinctly as grayish, firm, irregular areas. The splenic pulp is very soft.

The tongue shows no changes.

The tonsils and pharyngeal walls are swollen and bluish, but there is no ulceration.

The esophagus is normal. The liver shows no changes. The gall bladder contains about one ounce of straw-colored bile. The pancreas is normal. The adrenals are large and soft.

The kidneys are very much alike. They are large, soft, and pale yellowish-gray. They seem to be bloodless. The cortical markings are indistinct and the portions between the pyramids stand out more prominently than other parts. There are some areas that are whiter than others. The cortex measures 1–1.5 cm. in thickness. The capsule strips readily, leaving a fairly smooth surface.

The ureters and bladder are normal.

The retroperitoneal lymph glands in the region of the kidney are large and pink.

The submaxillary glands on both sides are as large as small hickory nuts and are quite firm. On the left side are some very soft lymph nodes containing pus.

Bacteriologic examination.—The streptococcus in pure culture was isolated from the pericardial fluid, the right and left pleural cavities, the spleen and the necrotic area of the face. The streptococcus and the colon bacillus were isolated from both lungs, the right kidney, the liver, and the peritoneal fluid.

Smears from the patient's internal organs showed neither spirilla nor fusiform bacilli; those from the tonsils showed fusiform bacilli, diplococci, spirilla. Smears from the cheek tissues at line of demarkation showed fusiform bacilli, spirilla, diplococci, streptococci, and smears from the suppurating submaxillary lymph gland contain streptococci.

Histological examination.—A section of the lung shows broncho-pneumonia with edema and hemorrhage. Other sections show the same changes but to a less degree.

There is marked hyperemia of the spleen.

The periportal connective tissue in the liver is very rich in cells.

The mesenteric lymph gland shows lymphoid hyperplasia.

The kidney shows collections of mononuclear round cells.

Part of the tissue removed from the cheek is completely necrotic, no nuclei being present (absolute alcohol, paraffin, hematoxylin, and eosin). Just beyond the advancing necrosis and in the living tissue there is some accumulation of leucocytes, but it is not marked. No sharply marked demarkation zone is present. The vessels are filled with red and white blood cells. In the areolar tissue of the necrotic and

infiltrated parts, faintly staining filaments, spirilla and pointed bacilli are seen. Some of the bacilli are much curved. The bacilli stain more deeply than the other organisms.

The bacilli, filaments, and spirilla do not retain Gram's stain, if the decolorization is thorough. The bacilli hold it longer than the filaments and spirilla.

Gram-Weigert sections show that under the external layer of the skin fusiform bacilli are present in considerable numbers in the normal tissue. The blood vessels in the normal and necrotic tissue are filled with red and white blood cells. Fusiform bacilli are present in large numbers in the walls and around the vessels. A moderate number of bacilli and filaments can be seen inside the vessels. The number of bacilli is small until the subcutaneous tissue is reached, but increases as the border between the healthy and necrotic tissue is approached. The bacilli stained by this method show very irregular staining. Some are considerably curved. They are often arranged in pairs and occasionally appear like filaments. No spirilla are seen when this stain is employed.

Carbol-gentian-violet was found to be the most satisfactory stain for the organisms. A 10 per cent solution of saturated alcoholic gentian-violet in 5 per cent carbolic acid was employed for five minutes, the section having been imbedded in paraffin and treated with xylol, followed by absolute and 95 per cent alcohol. After staining for five minutes, the specimen is cleared by means of aniline oil, washed with three changes of xylol, and mounted in balsam. When this method is used the spirilla stain clearly. The specimens were found much more satisfactory than when carbol-fuchsin was employed for 24 hours. The bacilli and filaments often stain more intensely than the spirilla, but in deeply stained preparations the three appear of the same thickness, with only an occasional thicker bacillus. In the area of complete necrosis fusiform bacilli, filaments, and spirilla are all present, the spirilla generally predominating in number. Where the infiltration begins one finds more spirilla and fusiform bacilli, the spirilla being more numerous than the filaments. In the living tissue both spirilla and bacilli are present, but the spirilla are in larger numbers. Spirilla are seen in some of the thrombosed vessels. In the areolar tissue where the organisms show most clearly, one finds a network of spirilla, in which can be seen a few fusiform bacilli and filaments. A large amount of fibrin is seen throughout the sections.

Summary of Findings.

I. There is some leucocytic invasion, but no well-marked demarkation zone.

II. The fusiform bacilli and spirilla are similar to those seen in the smear preparations made from the nose and mouth before death and the necrotic tissue of the face immediately after death.

III. Both forms are present in both the necrotic and living tissue, the spirilla forms apparently being in excess in both places.

IV. The thrombosed vessels contain fusiform bacilli, filaments, and spirilla.

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PLATE 1*a*.

Section through the living subcutaneous tissue just beyond the advancing necrosis. About ×800.

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THE VIRULENCE OF DIPHTHERIA ORGANISMS IN THE THROATS OF WELL SCHOOL CHILDREN AND DIPHTHERIA CONVALESCENTS.*

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IN spite of the lowering of the diphtheria death-rate due to the use of diphtheria antitoxin, and the immunity conferred upon those exposed to the disease when this substance is injected at a sufficiently early date, the fact still remains that the number of cases of diphtheria in all large cities is discouragingly high with a tendency to increase rather than to decrease. This increase in the number of reported cases is undoubtedly due very largely to the vigilance exercised by the more and more carefully organized boards of health, as indicated by the statistics of this city for the winters of 1902–5 inclusive. Here we find, in 1904–5, an increase in the number of cases reported with a marked decrease in the death rate. Expressed in tabular form the figures for the city as a whole are as follows:

TABLE 1.

Nov. 1 to March 1	Cases	Deaths	Mortality as per cent
1902–3	798	127	16.0
1903–4	1,254	204	16.3
1904–5	1,517	193	12.7

It is impossible, at the present time, to assign an exact explanation for the decrease in deaths in 1904–5, but the most probable reason is the more extended and careful oversight of school children, who are now visited daily by medical inspectors, and a wider use of laboratory methods of investigation in cases of throat inflammations. Yet, with all the oversight that modern methods can give, outbreaks of the disease occur which cannot be well controlled, and sporadic cases crop up in all classes of society in which it is frequently impossible to locate the original source of infection.

It is generally agreed[1] that a small percentage of healthy persons

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[1] For thorough discussion of this subject see: J. A. Schabad, *Jahr. Kinderheil.*, 1901, 54, p. 381; G. S. Graham-Smith, *Jour. Hyg.*, 1903, 3, p. 217; *ibid.*, 1904, 4, p. 258; *Report of Mass. Association of Boards of Health*, on "Diphtheria in Well Persons," 1902; Alice Hamilton, *Jour. Infect. Dis.*, 1904, 1, p. 690.

harbor virulent diphtheria organisms; it is also undoubtedly true that a much larger percentage harbor organisms corresponding morphologically to the Klebs-Loeffler bacillus but which are not actively virulent for guinea-pigs. Whether these non-virulent or slightly virulent organisms are responsible for the transmission of diphtheria is a question upon which there is a voluminous and contradictory literature.

For the practical necessities of boards of health the question of the supervision of young school children as carriers of diphtheria is of the greatest importance. Thomas[1] states that 85 per cent of those children to whom infections might be traced were between five and eight years old, a period when the child is not subject to the sharp, rapidly developing form of the disease, as are younger children, but is apt to have a mild or even unrecognized attack during which it is not isolated and is, therefore, a fertile source of trouble.

Another difficult problem of the health officer is that of the release of the patient when he is recovered, or of the removal of the quarantine from his dwelling. Here the family, and often the physician, comes into conflict with those boards of health which require that the patient's throat shall be microscopically free from the diphtheria bacillus before the quarantine is removed. The physician states that the patient is quite well and longer isolation is therefore unnecessary. Sometimes he asserts that, though the dwelling is still quarantined, the patient is associating freely with members of the family and no evil results follow his harboring the disease germs.

For the purpose of collecting additional data upon these two questions—the virulence of diphtheria organisms in the throats of well school children, and the virulence of the organisms from a clinically well diphtheria convalescent—certain studies, which are outlined below, have been made in this laboratory.

THE VIRULENCE OF DIPHTHERIA BACILLI IN THE THROATS OF WELL SCHOOL CHILDREN.

During the months of January and February, 1905, cultures were taken by the majority of the 50 medical inspectors of the city of Philadelphia from the apparently normal throats of 375 public-school children. Of this number 40, or 9.3 per cent, showed, on microscopic examination, bacilli corresponding in morphology and staining peculiarities with Loeffler's bacillus.

[1] *Brit. Med. Jour.*, 1904, 2, p. 431.

Pure growths of these bacilli were obtained by sowing a small amount of the original culture in the water of condensation which formed in the bottom of a large tube containing a serum slant. This water, with its suspended organisms, was allowed to flow once over the surface of the serum and the tube was then placed upright in an incubator. From the comparatively few colonies appearing after 18 to 24 hours other sowings were made in a similar fashion until purity was obtained. Several colonies were transferred from tube to tube in order to insure the final presence of various varieties of the organisms if they existed in the original culture.

In 37 cases the organisms so obtained were inoculated into guinea-pigs. Most frequently the first culture received was worked up; occasionally, when the microscopic examination indicated the persistence of the germs, a second throat culture was independently inoculated to determine whether the virulence of the type had undergone any change. In every case tried the results of the second inoculation corresponded with those of the first. The inoculations were subcutaneous, using the growth scraped from a 24-hour old serum culture.

An endeavor was made to examine these throats until three consecutive negative results were obtained. In only eight cases was this successful, the others furnishing positive results constantly, or very rarely negative. For some of the children the period of observation extended over 40 days; the average time for all the cases was 23 days.

Out of 37 inoculations made in duplicate 13, or 35 per cent, failed to kill either pig, though some of them lost weight. Of the remaining 24 five killed both animals promptly. Nineteen killed one of the two pigs after a period varying from 3 to 14 days, the other pigs surviving though there was a marked loss in weight and frequently a pronounced edema at the site of inoculation. On these pigs, as well as on those in which the infection terminated fatally, the wound healed slowly, though at autopsy the organism was frequently found quite pure.

An examination of the animals which died promptly gave a fairly typical picture of a diphtheritic infection; when they survived for from 10 to 14 days, or, in a few instances, even longer, the edema at the site of inoculation had largely disappeared and a small amount of inflammation remained. This was often covered by a gray, necrotic deposit that extended over the originally edematous area. In this membranous deposit at the site of inoculation the original organisms could invariably be found. They were not found in the body fluids nor in any of the organs. The kidneys were usually hyperemic, occasionally the capsule was slightly adherent, the suprarenals were always much injected and often the heart was greatly enlarged with gorged arteries or a fatty degeneration.

Reference to the protocol which follows will show that 19 of the 50 medical inspectors had one or more positive cases, the maximum for an individual being six. Twenty-five schools were represented and nine of them had more than one pupil showing these organisms. Of the 40 cases examined one child had been in undoubted contact with a diphtheric child next door; seven cases had diphtheria in the immediate neighborhood of their dwellings; 10 had a history of diphtheria in the same school either at the time the first culture was taken or within a short period of it. One case developed clinical signs of diphtheria, but the organism failed to kill guinea-pigs though it was tried twice. Another case had had "tonsilitis" about the time of the first examination, but bacteria obtained from this throat proved non-pathogenic.

TABLE 2.

Serial Number	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculation	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
29,872	8 F. D. H.	1–5–05+ 1–7–05–	Two pigs; lost weight, but recovered after 6th day	Nichols' Annex	None	None	None	Two infants at home	Deafness of patient
29,891	M. P. B.	1–5–05+ 1–8–05+ 2–1–05+	Not inoculated						
29,900	M. E. S.	1–5–05+ 1–7–05+ 1–31–05+	One pig gained steadily for 17 days; the other lost wgt. slightly	Powers School	.....	None	None	Two brothers in Powers' School	Had clinical evidence of mild case of diphtheria before first culture was taken
29,926	6 M. G. R.	1–6–05+ 1–10–05+ 2–10–05+ Organism isolated from 2 cultures and separately inoculated	Four pigs; 3 died. Average time 5 days. Other pigs sick, but pulled through	Muhlenberg School	None	None	Three cases in 3 houses opposite the school. One case fatal	Five children; all attend this school; 1 baby at home	No known contact between this child and cases cited; throat normal. Had *impetigo contagiosum* on chin
29,917	6 M. W. F.	1–6–05+ 2–10–05–	Two pigs; sick for a few days, but recovered	Clay School	Two cases between September and January. One child excluded because of case in family	Sister had "sore throat" with membrane and positive culture	One case half a block from home	Sister in this school; 2 in Weccacoe school. 2 infants at home	Out of school "because of illness" for a couple of weeks. Complained of sore throat when he retuned
29,936	6 F. G. K.	1–6–05+	Two pigs; lost weight slightly	Wm. McClellan School	None	None	None	One brother and 4 sisters in this school; 2 sisters doing mill work	
30,022	8 M. A. H.	1–6–05+ 2–4–05– 2–7–05+	Four pigs; not affected	Adams School	None	None	Brother in this school	.....	Headache and coated tongue
30,017	7 F. M. T.	1–7–05+ 2–7–05+ 2–8–05+ 2–9–05+ 2–10–05– 2–11–05+	Four pigs; not affected	James Lynd School	None	None	None	.....	Associated with child in adjoining house who, 1–16–05, had "sore throat" with 2 positive cultures, but was not quarantined. 2–8–05 this child's throat was clear

TABLE 2.—*Continued.*

Serial Number	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculation	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
30,008	M. E. D.	1–7–05+ 2–9–05– 2–10–05+	Two pigs; neither much affected	Bartram School	One case, about the time the first culture was taken	None	About one mile away	Sister in Kindergarten School, and 2 brothers in Morton and Bartram respectively	
30,076	12 M. R. W.	1–10–05+ 1–11–05+ 1–31–05+ 2–7–05–	Two pigs; died in 3 and 7 days respectively	Northwest School	None	None	None	None	
30,089	9 M. R. S.	1–10–05+ 1–31–05– 2–10–05– 2–11–05–	Two pigs; 1 gained, other did not	Francis Reed School	None	None	None	One brother, 11th and Moore St. School; 1 sister, 7th and Dickinson St. School	
30,090	10 M. S. M.	1–10–05+ 2–1–05–	Two pigs; 1 died, 1 recovered	Ramsay School	None since 12–23–04	None	None	Three children all in Ramsay School	
30,088	4 F. E. G.	1–10–05+ 1–11–05+ 1–17–05+ 1–19–05+	Two pigs: 1 died in 3 days, other survived	New Street School	One case	.....	.....	Brother and sister in New Street Primary	
30,105	F. H. M.	1–10–05+ 1–11–05– 2–9–05+	Two pigs; 1 died on 8th day, other surviv'd	Shippen School	None	None	None	One brother in Northwest School	
30,109	F. M.DeG.	1–10–05+ 1–14–05– 2–8–05–	Two pigs; not affected	Washington School	None	None	None	Five children in Washington School; 1 sister in factory; 1 brother in poolroom	
30,149	6 M. T. B.	1–11–05+	One died 13th day; other survived	Horace Binney School	None	None	None	Infant brother and sister at home	
30,204	M. H. S.	1–12–05+ 1–17–05+ 2–9–05+	Two pigs; died on 7th and 8th days respectively	New Street Primary	One case	None	.....	Three brothers in New Street Primary	

TABLE 2.—*Continued.*

Serial Number	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculation	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
30,195	11 M. G. S.	1–12–05+ 2–2–05+ 2–8–05–	Two pigs; one died on 11th day, other sick	Ramsay School	Not since 12–23–04	None	None	One brother in Ramsay School	
30,206	5 F. S. Z.	1–12–05+	Two pigs; one died in 9 days, other sick	New Street Primary	One case	.....	.....	Only child	
30,257	M. J. C.	1–12–05+ 1–19–05– 2–8–05+ 2–10–05+	Two pigs; not affected	Boon's Dam	Yes; about the time of first culture	None	Half a mile away	Three brothers in shops and a foundry	
30,257	M. D. R.	1–13–05+ 2–2–05– 2–8–05– 2–9–05– 2–10–05–	.....	Boon's Dam	Yes; about the time the first culture was taken	None	Half a mile away	Two children in Boon's Dam School; brother in soap factory; sister at home	
30,319	L. V.	1–14–05+ 1–19–05–	Two pigs; not affected	Bartram School	Yes	None	None	Brother in factory	
30,324	6 M. F. M.	1–14–05+ 2–8–05– 2–9–05– 2–10–05– 2–11–05–	Two pigs; one died on 17th day	A. D. Bache	None	None	None	Brother and sister; one in Bache School	
30,328	7 F. T. J.	1–14–05+ 1–18–05+ 1–20–05+ 1–22–05– 2–7–05+ 2–8–05– 2–9–05+ 2–10–05– 2–11–05– 2–14–05–	Two pigs; died on 14th and 17th day, respectively	Baldwin School	Three cases on following dates: 12–21–04 12–28–04 1–16–04 Not, however, in this child's room	None	Three in immediate neighborhood	Only child	When first culture was made had fever and sick stomach; chronic catarrh and atrophied tonsils
30,393	7 M. W. McK.	1–17–05+ 1–31–05+ 2–9–05+ 2–11–05– 2–14–05+	Two pigs; neither affected	Child's School	None	None	Four cases in immediate neighborhood	One brother in this school; baby at home	Had acute tonsilitis in January; on Feb. 8 he states that "now the baby is ill"
30,385	9 F. M. J.	1–17–05+ 1–31–05+ 2–2–05+ 2–8–05+ 2–10–05–	Two pigs; neither affected	Agnew School	None	None	None	One brother in this school	

TABLE 2.—*Continued.*

Serial Number	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculation	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
30,408	7 M. W. Y.	1–17–05+ 2–1–05+ 2–4–05+ 2–8–05– 2–14–05+ 2–17–04+	Two pigs; one died on 5th day, other nearly died	Ramsay School	None	None	None	One sister in this school	
30,488	7 F. J. C.	12–17–04+	.....	Jackson School	None	None	One case near dwelling	One sister in this school	
30,489	8 F. R. M.	1–18–05+ 2–1–05– 2–8–05– 2–10–05– 2–11–05– 2–12–05–	Two pigs; neither affected	Morton School	None	None	Two cases near dwelling	One sister in Morton School, another in Morton McMicheal School	
30,498	5 F. A. O.	1–18–05+ 2–1–05– 2–8–05– 2–10–05– 2–11–05– 2–12–05–	Two pigs; neither affected	Morton Primary	None	None	None	One sister in Morton School	
30,504	6 F. B. G.	1–18–05+ 1–30–05– 2–5–05– 2–7–05–	Two pigs; neither affected	Adams School	None	None	None	Brother in this school	
30,505	M. F. McM.	1–18–05+	Two pigs; not affected	Benson School	None	None	None		
30,509	6 F. E. K.	1–18–05+ 1–20–05– 2–7–05+ 2–8–05– 2–10–05–	Two pigs; both died on 6th day	Geo. L. Horn School	None	None	None	Three brothers and 1 sister doing mill work; 3 sisters at home	On 1–19–05 a child was excluded from this school because his brother had membranous croup
30,515	M. E. E	1–18–05+ 1–31–05+ 2–2–05– 2–7–05+	Two pigs; not affected	Nichols' Annex	None	None	None	One sister in Nichols School	Had tonsilitis about time first culture was taken
30,540	7 M. H. M.	1–19–05+ 1–23–05– 1–31–05– 2–9–05–	Two pigs; one died 14th day, other lost weight	Levering School	None	None	Brother and sister in department store and iron foundry; 2 children in this school		

TABLE 2.—*Continued.*

Serial Number	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculation	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
30,542	7 F. S. R.	1–19–05+	Two pigs; 1 died on 12th day, other lost weight						
30,901	9 M. A. R.	2–9–05+ 2–11–05—	Two pigs; not affected	Muhlenberg School	None	None	See 29,926	See 29,926	Brother of case 29,926
30,966	7 F. U. K.	2–11–05+	Two pigs; one died on 11th day	.....	.....	.....	.....	.....	Companion of cases 30,901 and 29,926
30,150	6 M. H. B.	1–11–05+ 1–14–05—	Two pigs; one died on 8th day, other lost weight for 5 days	Horace Binney School	None	.....	.....	One sister in this school	
30,159	7 M. H. R.	1–11–05+ 1–18–05— 1–25–05—	Not inoculated	Horace Binney School	None	None	None	Infant brother and sister at home	

It has seemed desirable in the prosecution of this study to examine simultaneously a few schools located in widely separated neighborhoods and attended by different classes of children, to determine, if possible, the relation of the presence of the organism to environment. Four schools were selected, all admitting both boys and girls and having an individual attendance of from 750 to 1,300 children. Cultures were taken from normal throats and examined as before, except that the inoculations were made subcutaneously with 1 c.c. of a 24-hour broth culture as well as with the serum growth in a skin pocket.

One hundred and twenty-five children were examined; 13, or 10.4 per cent, showed, on microscopic examination, the presence of diphtheria organisms. Those harboring the bacilli were distributed among the four schools as follows;

Kane School	2.5 per cent
William Cramp School	9.7 "
Mantua School	14.8 "
Geo. M. Wharton School	18.5 "

There is indicated here a direct ratio between the social condition of the children and the prevalence of the organism in their throats. The Kane School, having about 800 boys and girls, is attended by cleanly, well-kept children, mostly of well-to-do parents; the Geo. M. Wharton School draws its 1,300 scholars very largely from among the Poles, Italians, and Russian Jews, who are crowded into most unsanitary surroundings. The other two schools show intermediate social conditions and the percentage of infected throats corresponds.

The general average for the four schools is 10.4 per cent of persons harboring the organisms; a figure agreeing closely with that found in examining children haphazard all over the city. A comparative table of results obtained in the two series illustrates this point more clearly.

	Four selected schools	Twenty-five schools at random
Organisms killing guinea-pigs	15.3 per cent	13.5 per cent
Organisms showing some pathogenicity	30.7 "	51.3 "
Organisms without virulence	53.0 "	35.1 "
Children infected	10.4 "	9.3 "

For the cultures from the throats of these children and the histories of those carrying diphtheria organisms I am indebted to the corps of medical inspectors, and especially to Drs. Coates, Cornell, Fretz, and O'Hara.

The Virulence of Diphtheria Organisms in the Throats of Convalescents.

The organisms obtained from the throats of 25 individuals clinically recovered from diphtheria were tested for virulence. Whenever possible they were isolated from the culture just preceding the condition required for release from quarantine. In several instances the organisms were isolated at different times during convalescence and their virulence tested for a diminution in activity. In only one case was such a loss of virulence noted.

Nine of the 25 cases were sent to the laboratory by the medical inspectors for release from quarantine. For the histories of the other 16 as well as the cultures used in this work I am indebted to Dr. B. F. Royer, chief resident physician at the Municipal Hospital.

Twenty-two of the 25 cases examined gave virulent organisms. In 15 of the 22 they were tested on their last appearance and in the others only a few days elapsed after

TABLE 3.

No. of Scholars Examined	Age, Sex, Name	Microscopic Examination of Cultures with Dates	Animal Inoculations	Name of School	Cases of Diphtheria in This School	Cases of Diphtheria in the Family	Cases of Diphtheria in the Neighborhood	Schools or Employment of Family	Remarks
	11 M. J. H.	2-23-05+ 3-3-05— 3-7-05— 3-8-05—	No effect	Wm. Cramp	None	None	None	One brother in same school; other a laborer	This child attended a Sunday school in which was a case of diphtheria about this time
31	H. F.	2-23-05+	No effect	Wm. Cramp	None	None	None	Sister in Taylor School; brother in this school	
	12 M. A. B.	3-1-05+ 3-3-05— 3-7-05— 3-8-05—	2 pigs; lost weight, with induration at site of inoculation	Wm. Cramp	None	None	None	Sister in a mill	
40	12 F. I. D.	3-8-05+ 3-10-05+	2 pigs; marked loss of weight	Kane	None	None	None	Sister in same school	Had sore throat a short time before; 8 cases of "sore throat" in this child's room in Kane school; 5 of these examinations showed streptococci but no Klebs-Löffler
	10 M. T. M.	2-27-05+ 3-14-05—	No effect	Geo. M. Wharton	None	None	None	Four brothers and 2 sisters; all working in various establishments and all over 14 years	Parent has very large tonsils.
	9 M. P. Z.	2-27-05+	No effect	Geo. M. Wharton	None	None	None	Four sisters; 2 in school. Mother recently had grippe	
	9 M. B. G.	2-27-05+ 3-14-05+	No effect	Geo. M. Wharton	None	None	.....	One sister; at house; no illness in family	Four famlies live in this child's house. Scarlet fever one year ago.
27	10 M. A. H.	2-27-06+ 3-14-05—	1 pig showed necorosis and loss of weight; other died in 3 days. Typical diphtheria	Geo. M. Wharton	None	None	.....	Sister and brother in this school; 1 brother in high school	Three families in this house. No illness at time of first culture.
	9 M. C. H.	2-27-05+	2 pigs died in 2 days	Geo. M. Wharton	.....	.....	.....	Two brothers, two sisters, all between 5 and 12 years; attend different schools. Father keeps saloon and boarding-house	
	8 M. C. W.	2-27-05+ 3-3-05— 3-6-05— 3-8-05—	No effect	Mantua	None	None	None		
	7 F. S. N.	2-28-05+ 3-3-05— 3-6-05—	No effect on guinea-pigs	Mantua	None	None	None		
27	7 M. F. R.	3-8-05C. 3-8-05+	Pigs lost weight; wounds necrotic	Mantua	None	None	None		
	6 F. C. M.	3-8-05+	Lost weight; wound did not heal for one month	Mantua	None	None	None		

TABLE 4.

Name and Serial Number	Microscopic Examination of Cultures with Dates	Animal Inoculations	Duration of Disease before First Culture for Disinfection Was Taken	Administration of Antitoxin	Remarks
V. H. 31,470	3–28–05— 3–29–05 C 3–30–05— 3–31–05+ 4–2–05— 4–3–05—	Two pigs, from culture taken 3–31–05; both dead in 3 days	Nine days	Satisfactory	
A. D. 31,463	3–18–05+ 3–21–05— 3–21–05—	One pig; died on second day; culture of 3–18–05 used	Fourteen days; clinical evidence gone on fifth day of disease	Satisfactory	
A. D. Jr. 31,464	3–18–05+ 3–21–05— 3–21–05—	One pig; died in 24 hours	Fourteen days	Satisfactory	
G. C. 31,436	3–16–05+ 3–17–05+ 3–21–05— 3–22–05—	Two pigs; culture of 3–16–05 used; both died in 2 days	Fourteen days; clinical evidence had disappeared on fifth day	Satisfactory	
C. O'C. 31,574	3–28–05— 3–29–05+ 3–30–05+ 4–1–05+ 4–2–05+ 4–3–05+ 4–5–05— 4–6–05—	Two pigs, inoculated from culture made on 3–28–05; died on second day. two other pigs inoculated from culture of 4–31–05 also died on second day. One pig, from culture of 4–2–05, died second day	Eight days; fifth or sixth day when clinical signs disappeared	Satisfactory	
S. S. 31,502	3–23–05 C* 2–24–05+ 3–25–05+ 3–26–05+ 3–29–05— 5–30–05 C	Two pigs; both died in 5 days; organism from culture on 3–24–05 Two pigs inoculated from culture taken 3–28–05 died in 2 days	Fourteen days	Satisfactory	
S. van L. 31,264	3–4–05+ 3–5–05— 3–7–05+	Two pigs inoculated from culture of 3–7–05; both died in 2 days	Seventeen days; clinical signs disappeared on tenth day	Satisfactory	
B. M. 31,176	2–27–05+ 3–1–05+ 3–3–05+ 3–4–05+ 3–5–05— 3–6–05 C 3–7–05+ 3–8–05—	Two pigs inoculated from culture of 3–7–05; both died in 2 days	Thirteen days; child had been well for a week	Not given	
W. M. 31,294	3–7–05+ 3–8–05+ 3–14–05 I 3–15–05+ 3–22–05— 3–23–05+ 3–24–05— 3–25–05—	Two pigs; died in 24 hours. Inoculated from culture taken on 3–8–05	Seventeen days; well for seven days	Satisfactory	
C. R. 30,212	3–6–05+ 3–7–05—	Two pigs, not affected	One hundred days; exudate disappeared on fifth day	Satisfactory	Admitted for diphtheria. Contracted scarlet fever while waiting for negative cultures
G. J. 31,259	3–16–05+ 3–17–05— 3–18–05— 3–21–05— 3–22–05—	Two pigs; died in 24 hours	Throat cleared up ninth day of disease		
R. W. 31,261	3–15–05— 3–16–05+ 3–17–05— 3–18–05—	Two pigs; died in 22 hours	Eleven days	Satisfactory	
R. R. 31,422	3–22–05+ 3–31–05— 4–1–05—	Two pigs; died in 24 hours	Eleven days	Administered	

*_B. subtilis_ had liquefied the serum, making examination for diphtheria bacilli impossible.

TABLE 4.—*Continued.*

Name and Serial Number	Microscopic Examination of Cultures with Dates	Animal Inoculations	Duration of Disease before First Culture for Disinfection Was Taken	Administration of Antitoxin	Remarks
A. L. 31,426	3–22–05+ 3–23–05– 3–24–05–	Two pigs; died in 24 hours	Eight days	Administered	
J. McA. 31,540	4–3–05+ 4–4–05– 4–5–05–	Two pigs; died in 48 hours	.....	Administered	
G. McA 31,543	4–3–05+ 4–4–05+ 4–5–05+ 4–6–05– 4–7–05+ 4–8–05+ 4–11–05+ 4–13–05+ 4–14–05– 4–15–05–	Two pigs; inoculated from culture made 4–3–05. Both died on second day	Sixteen days; well for ten days	Administered	
A. McA 31,544	4–3–05+ 4–4–05– 4–5–05+ 4–6–05+ 4–7–05+ 4–11–05+ 4–12–05– 4–13–05–	Two pigs; died in 24 hours; culture of 4–3–05 used	Fifteen days; no clinical signs for eight days	Administered	
J. C. 31,349	12–16–04+ 12–21–04– 12–22–04+ 12–23–04+ 12–24–04+ 12–26–04+ 12–27–04+ 12–28–04+ 12–29–04+ 1–4–05– 1–8–05+ 1–9–05+ 1–10–05+ 1–12–05– 1–13–05– 4–3–05+	Two pigs; not affected	One hundred and tenth day when cultured	Administered	A nurse in the diphtheria wards. Has continued to give an occasional positive culture for months after complete recovery from an attack of the disease
H. O.	3–17–05+ 3–31–05– 4–3–05– 4–4–05–	Two pigs; died in 48 hours	Seventeen days	Administered	
K. R. 31,412	3–15–05– 3–16–05– 3–17–05+	Two pigs; died in 48 hours	Six days	Administered	
E. R. 31,417	3–12–05+ 3–13–05+ 3–17–05+ 3–20–05+ 4–3–05– 4–4–05–	Two pigs; inoculated from culture obtained 3–20–05. Died in 24 hours.	Twelve days; well for seven days	Administered	
F. S. 31,587	4–3–05+ 4–7–05– 4–9–05– 4–10–05–	Two pigs; killed in 24 hours	Twelve days; no exudate for six days	Administered	
A. G. 31,585	4–3–05+ 5–4–05+ 5–6–05+ 5–7–05– 5–8–05–	Two pigs; died in 48 hours. Culture of 4–3–05 used	Fourteen days; no exudate for four days		
R. T. 31,593	4–3–05+ 4–14–05– 4–15–05–	Two pigs; both died on second day	Thirteen days; no exudate for six days		
J. A. 31,202	2–28–05+ 3–7–05– 3–8–05+ 3–18–05+ 4–4–05+*	One pig; died in 3 days; culture of 3–18–05. Two pigs inoculated with culture of 4–4–05 were not affected	Two consecutive negative cultures were never obtained; patient discharged when organism ceased to show virulence. Exudate disappeared on 5th day	Administered	

*Continued positive until 5–19–05 when patient was discharged, eighty-fourth day.

the cultures had been taken before the throat was free of them. These final positive cultures were made from 5 to 17 days after the patients were recovered from the disease, the average time being nine days.

Two cases showed diphtheria organisms morphologically perfect, but they did not affect guinea-pigs. One of these patients was a nurse in the Municipal Hospital who contracted the disease and was released from quarantine on the 28th day, two consecutive negative cultures having been obtained. Frequently, however, cultures from her throat were found to be positive (see case J. C. 31,349), but the organisms when tested on the 110th day lacked virulence. The second case contracted scarlet fever while waiting for the organisms to disappear from his throat, all other signs of diphtheria having vanished. After complete recovery from scarlet fever the presence in the throat of organisms morphologically identical with Loeffler's kept him at the hospital for some weeks. One hundred days after all clinical signs of diphtheria were gone the organisms were isolated and inoculated into guinea-pigs. They were not active.

Case No. 31,202, J. A., gave 18 days after recovery a virulent organism. Microscopically the organisms persisted and were again tested 17 days later. This test showed absolutely no virulence. The case was kept under observation for 45 days longer, but the organisms had not, at the expiration of that time, disappeared.

SUMMARY.

Among well school children approximately 10 per cent harbor in their throats bacilli which correspond morphologically with the organisms of diphtheria.

One-half of these organisms are without effect on guinea-pigs. About 30 per cent behave like attenuated forms and 14 per cent kill the animals with a fair degree of promptness.

Of 25 strains of diphtheria bacilli obtained during the last days of convalescence 23 were highly virulent; 2 were without virulence, one being from the throat of a convalescent nurse constantly associating with diphtheria patients, the other from a case which had contracted scarlet fever while convalescing from diphtheria. One case gave a virulent organism on the 18th day and a non-virulent one on the 35th day.

In the report of the Massachusetts Association of Boards of Health on "Diphtheria Bacilli in Well Persons" is found the following paragraph: "As there is no sharp line to be drawn between the healthy and the diseased state, one shading imperceptibly into the other, so there is no sharp line to be drawn, for many infections at least, between the time when the micro-organisms are still in the body and when they have all been destroyed or eliminated. It follows logically, as is shown by the work of certain members of this committee, that

well persons may be at times the source of infectious diseases." In this very admirable paper the conclusion is also stated that the organisms in the throats of well persons recently exposed to diphtheria are likely to be virulent.

Such observations naturally lead to the consideration of the question of the increase or decrease in the virulence of the same strain of the diphtheria bacillus depending upon its environment. An absolute differentiation of the so-called pseudo-diphtheria bacillus from the diphtheria bacillus has not yet been accomplished. On the other hand, we meet with recent evidence that morphologically and clinically the most satisfactory pseudo-forms have a decided pathogenicity for guinea-pigs and, from clinical evidence, for human beings;[1] hence the harmlessness of these germs cannot be accepted unconditionally, any more than a certain virulence can be attached invariably to a diphtheria bacillus which agrees with some arbitrary requirements laid down by bacteriologists.

As long ago as 1890[2] Roux and Yersin showed that virulent diphtheria bacilli could be made quite harmless by an unfavorable environment and that a non-virulent organism, acting on a guinea-pig in conjunction with an organism of quite a different variety, such as the streptococcus of erysipelas, will become highly pathogenic.

More recently[3] Ohlmacher not only heightened the virulence of the bacillus by passage through a guinea-pig, but changed the morphology of a short, solid form to the slender curved variety; and conversely, inoculation of a granular form into a white rat changed the organisms to those having the short, pseudo-morphology.

Additional laboratory data to determine exactly the biological relation, if there be such, between the virulent and non-virulent diphtheria organisms is still greatly to be desired. Clinically it would seem to be fairly well established that well persons harboring diphtheria bacilli who have not been recently exposed to the disease, are occasionally responsible for a true diphtheric infection, but such cases are rare. It is also true that the organisms found in the throat of a well person not exposed are, in the majority of cases, without virulence.

[1] Alice Hamilton, *Jour. Infect. Dis.*, 1904, 1, p. 670.

[2] *Ann. de l'Inst. Pasteur*, 1890, 4, p. 384.

[3] *Jour. Med. Res.*, 1902, 7, p. 128.

Those well persons who have been exposed to diphtheria and who carry the organisms in their throats, while perfectly well themselves, do, very frequently, harbor an organism of marked virulence and are responsible for many more infections than are the well, unexposed persons. Lastly, the convalescent from true diphtheria will carry and disseminate virulent organisms so long as any remain in his throat, which period may far exceed the duration of the clinical evidences of the disease.

It is required of those having in charge the public health that the majority shall be protected from disease with the minimum amount of inconvenience to the minority who are responsible for the spread of any infection. What these protective measures shall be must depend not only upon the infection to be combated but, very often, upon the conditions prevailing at the moment. The isolation of a clinically well diphtheria convalescent, while waiting for the organisms to disappear from the throat, frequently causes inconvenience and even hardship, but from the accumulated evidence on the subject there seems but one course to pursue, namely, to forbid association with non-immunes so long as the laboratory findings are "positive."

What course should be taken in regard to the well persons not exposed to diphtheria who carry bacilli is not so clear. To quarantine such a number would be impractical; yet the bacteriologist must insist that, in the absence of definite evidence to the contrary, the organism which is harmless and attenuated in the throat of a well individual may, if planted on another throat under different conditions of susceptibility, so increase in virulence that its specific disease is produced.

The best argument for the infrequency of such an increase in virulence, if, indeed, it ever happens, is the fact that so many well persons carry the organisms without apparent menace to their associates. In the case of school children, if medical supervision is possible, it would seem desirable that they be watched carefully for any clinical manifestations of diphtheria and that they should be taught personal habits which will render infection of others less likely.

RELATION OF THE *BACILLUS MUCOSUS CAPSULATUS* GROUP TO RHINOSCLEROMA, AND OF THE VARIOUS MEMBERS OF THE GROUP TO ONE ANOTHER.*

ROGER G. PERKINS.

(From the Pathological Laboratory of Western Reserve Medical School, Cleveland, Ohio.)

DURING a former study of the so-called *B. mucosus capsulatus*, published in this *Journal* in 1904, questions concerning the relation of the variety known as the rhinoscleroma bacillus to the disease of that name, came prominently into view. Absence of material other than cultures from outside sources made further investigations impracticable, until the appearance of an undoubted case in Cleveland in 1905.

The literature on the group of organisms and on the disease of rhinoscleroma is very extensive, and has been too often reviewed to make a discussion necessary here. The most comprehensive summary is that of Hasslauer,[1] and a study of his article and of others shows certain points of universal agreement and some points concerning which various authors markedly disagree.

The disease itself is a proliferative lesion of the upper respiratory tract, often extending as low as the larynx. It is confined to man, occurs endemically in eastern Austria and adjacent countries, sporadically in Europe and Asia, and even less frequently elsewhere. The vast majority of cases occur among Slavs. It is apparently non-contagious, or very slightly contagious, for sporadic cases in new places are apparently imported and do not set up foci of infection. Further, with one or two doubtful exceptions, experiments in transmission to animals have been uniformly unsuccessful.

The lesions are in the form of soft, grayish masses which may reach large size, and on histological examination are found to be of the infectious granuloma type, made up of new-formed vascular granulation tissue with a varying amount of old and new fibrous tissue. Near the surface, and, to a less extent, deeper in the lesion,

* Received for publication September 5, 1906.

[1] *Centralbl. f. Bakt.*, 1904, 37, p. 1.

are hyaline areas, many of which contain masses of bacilli, singly or in pairs, often with well-marked capsules. These areas are said by most writers to be cells which have undergone degeneration, while some consider them to be lymph spaces. These areas are usually known as Mikulicz cells, and held to be histological characteristics of the disease.

The presence of organisms in the tissues led to extensive cultural experiments, with the result that in all cases of the disease bacilli of the mucosus capsulatus group were found. Before bacteriological differentiations reached their present detail, no discrepancies in the various reports were observed, but review of the descriptions of cultural characteristics shows that the organism noted as essential to the disease has varied from a fairly active fermenter to an absolute non-fermenter, and that the action on milk and the pathogenicity have also been variously described. At present the organism most often described as the rhinoscleroma bacillus does not form gas in sugar media, does not coagulate milk, and is of variable, but in general feeble, pathogenicity.

The case which afforded material for this paper came to Dr. W. H. Marvin, of Cleveland, in the spring of 1905. The patient was a Slav from eastern Austria, 35 years of age, and had suffered from the disease before his arrival in America two years previously. The growths, which were in every way typical, had caused so much narrowing of the larynx that tracheotomy was necessary. At the time of the operation Dr. W. T. Howard, Jr., was asked to be present, and it is to his courtesy and to that of Dr. Marvin that I am indebted for my material.

At the time of the operation cultures were made from the surface of the growths in the nose, and immediately afterward from deeper parts after suitable aseptic precautions. A large number of plates were made from these cultures, and the growths themselves were preserved for microscopic examination.

Coverslips from the inside of the nose, from the surface of the growths, and from the depths of the lesion showed varying numbers of short bacilli mostly in pairs, with well-marked capsules, a few cocci, and a few slender uncapsulated bacilli. All except the cocci decolorized by Gram. The plates from the different sources gave

practically indistinguishable growths, except that those from the depths of the lesion were almost free from cocci; the characteristic colony was large, flat, transparent, sharply defined, with a moist appearance, and on coverslip showed capsulated diplo-bacilli. The cocci noted in the coverslips were identified as the ordinary pyogenic forms, and the non-capsulated bacilli were found to be actively motile, and were later identified as colon bacilli.

Of the non-motile, capsulated forms, 12 cultures were selected on account of slight differences in the appearance of the colonies. These were denoted by letters, as were also such organisms as were studied for comparison later. These 12 were inoculated into the usual media and into fermentation tubes, with the result that they fell at once into three groups, one, including *C*, *E*, *F*, *G*, *K*, *M*, *N*, and *X* answered to my *Bact. aërogenes* group, fermenting dextrose, lactose, and saccharose with gas formation, a second group *A* and *B*, fermenting dextrose only, and a third, *D* and *Z*, apparently unable to form gas in any of the sugars. As a result of this preliminary work, *E* and *X* were taken as representatives of Group 1, *A* of Group 2, and *D* and *Z* of Group 3.

During the course of the work several organisms of the group were obtained from various sources and cultivated by way of comparison. Of these strains, *O* and *S* were grown from cases of turbinate hypertrophy, in no way related to rhinoscleroma, and *R* was found in a guinea-pig confined with other pigs inoculated with members of the series. As a further comparison, cultures of Edward's *Bacterium mucogenum* were obtained, one in 1905, one in 1906, through the courtesy of Dr. W. W. Ford of Johns Hopkins.

The present paper treats of the cultural reactions and the differentiation of these organisms from one another and from organisms of the same group obtained from other sources, of the relation of the bacteria in the tissue to the lesions, and presents a general discussion of the group. These points will be dealt with separately.

Bacteriological technique.—The routine laboratory media, used fresh and slightly alkaline to litmus were found to give very favorable growths. In the fermentation tests, however, dextrose-free broths were used as a basis, with dextrose, lactose, and saccharose in the proportion of 1 per cent for differentiation. The dextrose was the usual powdered glucose, but the lactose and saccharose were tested to measure their freedom from dextrose. Merck's and Kahlbaum's were found satisfactory.

The broth was titrated to show a faint rose pink with phenolphthalein, and after fractional sterilization the reaction was found to be from +0.5 to +0.10, and the medium gave very active gas formation with fermenters from other groups. Each batch of media was tested with the stock culture of *B. aërogenes* as a control, and unless gas appeared in 24 hours, the batch was rejected.

It was found that sealing or capping the mouths of the tubes stimulated contamination by mold, so that it appeared to be more satisfactory to refill the bulbs from time to time with sterile distilled water. Whenever a series was refilled, 5 c.c. samples of the water were placed on agar, in broth, and in dextrose fermentation tubes and in no case was there any growth, thus proving the sterility of the water, a point the great importance of which will appear later.

The usual stains were tried, and the capsules were easily demonstrated by any of the current methods.

Histological.—The tissue was hardened in Zenker's and alcohol, cut in paraffin, and stained with hematoxylin eosin, eosin and methylene blue, Giemsa's stain, and Weigert's.

General characteristics.—Cultures were made on plain, glycerin, and dextrose agar, in gelatin, broth, and litmus milk, and on potato. The most conspicuous thing about the *agar cultures* with the exception of *A* and *B*, was the transparency of the growth, which has been noted by practically all observers in cultures from this disease. The streak, after 24 to 48 hours was broad, raised, almost the color of the agar, and in certain lights could hardly be seen. This was no more marked in *D* and *Z*, the non-fermenting varieties, than in the forms such as *X*, which fermented all three sugars. Growth in the stab was uniformly good, and in the dextrose fermenters gas was seen in the dextrose agar. The water of condensation was thickened, and usually viscid, though this viscidity varied with different strains and even in different sets of media, being most marked in *E* and least in *X*. The cultures *A* and *B* were more of the ordinary type, far whiter and more porcelain-like, with practically no viscidity. The growth on the slant in the more active strains had a tendency to show infolding at the edges, which, on account of the differences of refraction, gave the appearance of a mixed culture, but plating showed no contaminations. As the cultures grew older, the edges faded more and more, until they became almost indistinguishable, with a rather whiter line in the center, and a white sediment in the water of condensation. With the exception of the distinction between *A* and *B* and the rest, the agar cultures were insufficient to distinguish the varieties.

GELATIN stabs showed no liquefaction after six weeks. Some of the varieties showed more nail growth than others, notably *A* and *B*, but this depended more on the concentration of the gelatin than on any other detail.

BROTH gave uniform cloudiness, more marked on shaking, with a heavy, often viscid precipitate, and frequently with a slight pellicle or a more or less marked white ring at the surface. Indol was slight in this series but present in all.

POTATO in general showed rather poor growth unless the medium was made distinctly alkaline with a calculated quantity of NaOH, when the growth was profuse, spreading, moist, and, in the case of the more active gas formers, showed occasional gas bubbles. After a week or 10 days the potato usually became discolored.

MILK. Change of reaction was slow in this medium. The organisms fermenting lactose gave acid reaction after a variable number of days (see Table 3), but never to a degree sufficient for coagulation. The non-lactose fermenters acted variously.

A gave sharp alkali from the second day, increasing in intensity but never reaching a grade sufficient for solution of the casein, while *D* and *X* gave very slight acid at first, followed by a return to neutrality, after which there was no further change.

In general the cultural results on routine media were sufficient to distinguish *A* and *B* from the others, but not for any further differentiation. This agrees essentially and in detail with the results in my preceding paper, and leaves in the way of ordinary cultures, nothing but the fermentation test. The results in this line will be taken up more in detail.

GLYCERIN MEDIA, while very favorable to the growth of the organisms, showed gas only with the most active strains, indicating weakness in activity toward glycerin, borne out later by the fermentation tubes.

Pathogenicity.—Inoculations into animals were made from time to time, but with no idea of classification by that means, as previous experiments with this group showed the variability of the pathogenicity to be so great as to make this unsatisfactory. Several members of the different groups were inoculated in varying amounts into guinea-pigs and mice, in an attempt to reactivate the feeble ones, and the results are best summarized as follows:

A was found pathogenic for guinea-pigs, in intraperitoneal inoculations of 1 c.c. in 48 hours, and the recovered organisms acted culturally exactly like the original.

D showed variable activity, in one case killing a guinea-pig with 1 c.c. in less than 24 hours, and another failing to kill with a dose of 10 c.c. The recovered organisms showed no change.

G was resisted in a 5 c.c. dose at one trial and was fatal to a guinea-pig in a 1 c.c. dose in another case. No change in organism.

M killed guinea-pigs in 48 hours, with no change in the organism recovered.

X apparently the most virulent, killed in 1 c.c. doses in a few hours.

Z was resisted by guinea-pigs in small doses, but proved fatal in large ones.

During this series a new organism was obtained from a guinea-pig which had been inoculated with tuberculous lymph glands, and owing to a lack of room was in the same cage with pigs inoculated with *D* and *Z* respectively. The organism recovered was culturally similar to *Z*, the animals had bitten each other, and it was supposed that transmission of infection took place that way. This organism was, however, given the name of *R*, and found readily pathogenic for guinea-pigs in small amounts.

The autopsies on all these animals showed very similar lesions. There was congestion and cloudy swelling of the organs, with peritonitis of a greater or less degree, usually somewhat viscid, and showing capsulated organisms of the original type. The lymph glands were enlarged, and in *M* and *D*, acute pleurisy, with presence of the organisms in the exudate, was found, while in *X* the lesion was of the acute hemorrhagic type, this variety being by far the most virulent of the lot. In all these the organisms recovered showed no changes in their cultural reactions.

Edwards' 05 (inactive and active), were pathogenic to mice in small amounts, and the organisms showed no special changes, except that Edwards' 05 (inactive), was more profuse and moist, approaching more closely the activated form.

Analysis of these results shows that practically all the organisms isolated from the case were more or less pathogenic for animals, without regard to their fermentative action, and further that different members of the same fermentative group reacted differently toward animals, so that classification on this basis would give pecu-

liar results. As far as could be seen, there was no increase of fermentation after passage through the animals, except possibly in the case of *R*, contrary to the findings in the former paper. It must be noted, however, that the cultures in this case were all direct from living tissues, while those noted before were old and decadent laboratory cultures.

It is important also to note that in no case was there any tendency to productive lesions, but the death of the animal occurred usually in two to five days, and the method of inoculation does not encourage the formation of that type of infection.

Fermentation tests.—The details of the different tests on dextrose, lactose, and saccharose may be found in the appended tables, but the results can be more clearly seen from a general discussion. It will be noted that in the table, *A* is used as representative of the group *A B*, because the two acted in an identical manner, but that although *X* was taken as representative of the group fermenting all three sugars, *E*, *G*, and *M*, of the same group, show differences which are worthy of note. In the same way *D* and *Z* are both recorded.

A was found to ferment dextrose quite readily, forming bubbles in dextrose agar in four to six days, and gas in the closed arm of the fermentation tube in 48 hours, while up to the end of 32 days there was no sign even of a cloud in the closed arm. These tests were made in the spring of 1905, and the stock culture of the organism died out in the summer so that no further tests could be made. I am therefore unwilling to state definitely that no fermentation would have occurred in further trials. The milk cultures were alkaline from the start, and there was marked alkalinity in the bulbs of the fermentation tubes, so it is at least probable that lactose was not fermented.

X, representative of the *Bact. aërogenes* group, fermented all three sugars with gas formation, but with markedly different reactions toward the different sugars, which variations were consistent. Dextrose was fermented in from 24 to 72 hours, while lactose required five to eight days, and saccharose eight to 22 days, in one case no fermentation of saccharose beyond the formation of a cloud being noted.

E, another representative of the same group, fermented dextrose in 48 hours to five days, lactose in 2 to 12 days, and saccharose in one case not at all, in other cases in 10 to 19 days. In neither of these strains was there any notable change during the different series, but in two other strains, *G* and *M*, there was an apparent loss of power, *G* fermenting dextrose readily in 48 hours throughout, but showing progressive slowness in saccharose from 2 to 11 days and then 20 days, while the lactose swung back and forth from eight to two to seven days. *M*, last in all the sugars, dextrose going from two to four days, lactose from two to five to seven days, and saccharose from 3 to 10 days, finally no fermentation taking place in 32 days.

D and *Z* consistently failed to form gas, though a cloud of singular persistence was seen in the closed arms of both the dextrose and saccharose tubes. At the end of 42 days the cultures were still alive, but no further change seemed to take place, the cloud remaining permanently.

Before going on to the results in the other group, it may be well to note certain details concerning two other organisms isolated from the nose in two cases of turbinate hypertrophy, the one, *S*, from the surface, the other, *O*, both from the surface and the depth of the mass. The cases were both chronic hypertrophic rhinitis of the usual type, the tissues in no way resembling those of rhinoscleroma. *S* was of the *Bact. aërogenes* type, as seen in the table, and fermented all three sugars readily in

24 hours, much as did the stock *Bact. aërogenes*. On the other hand, *O*, while it made gas readily in dextrose and glycerin agar, took 6 to 12 days to form gas in dextrose fermentation tubes, gave no change in 25 days in lactose or saccharose, made milk sharply alkaline, and failed to make gas aerobically in 1 per cent saccharose agar. In growth and in morphology it closely resembled *A*. This organism was obtained late in the year, and repetition of the fermentation was not possible on account of lack of time, but it appears to be a dextrose fermenter only.

Here then we have a series of organisms fermenting in varying degrees, and each one varying according to the suitability of the medium. *D* and *Z* form a cloud in dextrose and saccharose, indicating some degree of fermentation, but not to the point of gas formation. *A* and *O* form gas in dextrose, but are apparently unable to break up the double sugars. In the others we have a progressive series of activity from *M* through *G* to *E* and then *X*, culminating in the very active *S*.

With this suggestive basis we come to the next series of fermentations. These deal with organisms of the mucosus capsulatus group supposed by others, or by myself, to be completely inactive toward all three sugars, more so even than *D* or *Z*. This set includes *R*, Edwards' 05 and Edwards' 06. As seen in the table, in the first two trials *R* showed not even a cloud in the stem, and similar claim is made by Edwards for his organism, *Bact. mucogenum*. The first two were inoculated routinely into fermentation tubes made up from dextrose-free broth which had stood in a sealed flask from May until January. The tubes were inoculated in the usual way and for nine days no change was noted. At that time, owing to evaporation, the bulbs were found to be nearly empty and were filled with distilled water, control inoculation being made as described under *Technique*. The next day a cloud was seen in the dextrose of Edwards' 05 and two days later in that of *R*. The next day gas appeared in *R*, increasing for two more days, up to which time the only change in Edwards' 05 was increased density of the cloud. Thirteen days after the filling-up with water, gas began to appear in Edwards' 05 dextrose, *21 days from inoculation*, and on this day there appeared in the saccharose of *R*, with a cloud in the lactose. This cloud increased the next day, with the formation of gas and the simultaneous appearance of gas in the lactose of Edwards' 05. The saccharose tube of the latter showed a marked cloud. *Forty-two* days after inoculation the saccharose of Edwards' 05 still showed no gas, but became infected with mold and had to be discarded.

These surprising activities were looked on with great suspicion as probable contaminations, and steps were taken to find out if such were present. Plates made from the dextrose of Edwards' 05 and of *R*, from the saccharose of Edwards' 05 and the lactose of *R*, showed in the first three cases contamination to a moderate degree with a spore-bearing bacillus, having a tendency to thread forms. The only other organism present besides the spore bearer in each tube was a short diplo-bacillus similar to the original. In the lactose of *R* only the diplo-bacillus was found. Isolation of the different species showed that the contaminations were not identical, practically excluding a common source. The diplo-bacilli grew out in large, round, moist colonies in which they were distinctly capsulated, and all the colonies were alike except those from the saccharose of Edwards' 05. Inoculations were made into dextrose fermentation tubes and into dextrose and glycerin agar, and it was found that the diplo-bacilli fermented with more rapid gas formation than any of the others previously noted, while the contaminating spore-bearers formed no gas in three weeks and were apparently purely aerobic in their growth. The diplo-bacilli from the saccharose

of Edwards' 05 did not form gas, but showed a more profuse and moist growth than previously.

At a later time the evaporating tubes of saccharose of *E* and *X*, which had not shown any gas formation, were refilled with sterile water, and a few days later gas began to appear. Plates from these showed pure cultures of the original organisms.

This series of results is the chief interest of the paper. To resume it in brief, we have first a series of fermentation-tubes nine or more days old, which have shown no sign of gas formation. On account of evaporation of the fluid in the bulb, the portion lost was replaced with water, proved sterile, and in a period varying from two to 14 days signs of activity appeared. Careful plating showed most of the tubes to be completely uncontaminated, and furthermore showed that such contaminations as were present were completely inactive as regards fermentation. The members of the *B M C* group found in the plates made from *E* and *X*, known as fermenters, showed no alteration from the originals when studied on the usual media, while those from *R* and from Edwards' 05 showed marked increase in activity, and an appearance on culture closely resembling the other members of the group.

Exclusion of the spore-bearing contaminations as agents in this active fermentation leaves us two alternatives, the first that the organism in question has become activated or reactivated, the other that there has been a contamination by a bacillus of the same group identical morphologically, but with fermentation and cultural characteristics not only unlike the organism originally inoculated in the tube, but even unlike any in stock before or since. Of the two possibilities, the former seems the more simple, but for further check another culture of Edwards' organism was requested from Baltimore, and on its arrival called Edwards' 06. Inoculation after several subcultures on glycerin agar resulted in gas formation in dextrose in 12 days, lactose in 14 days, and a cloud in saccharose, very similar to what occurred in the Edwards' 05. As a further check, Edwards' 05 was retested, and though after four weeks there was a cloud in the tubes, no gas was found, but after eight weeks (56 days) gas was formed and plates showed pure cultures, checked by coverslips and cultures on various media.

We then have *three separate cases* of reactivation, and, so far as I can see, all possibility of contamination has been removed. The discussion of the conclusions will be postponed to a later part of this article.

The cultural reactions of these reactivated organisms showed close similarity to the type of the rest of the group, and the growths in media containing sugar were of course modified. Acid was formed in milk in a few days, and gas appeared in glucose agar. A possible explanation for the absence of coagulation in milk, in spite of the active fermentation of lactose is that the alkali formation was still sufficiently active to keep the acid below the clotting percentage.

All the organisms of this series showed inability to ferment glycerin in 1 per cent solution in fermentation tubes, although the stock *Bact. aërogenes* gave one-fourth gas in 24 hours, with subsequent increase. In the former paper it was noted that the old laboratory cultures reactivated by passage through animals showed their greatest loss of power in their action on glycerin.

Acid formation.—Bertarelli,[1] in a recent paper, has classified the organisms of this group according to their acid formation, and a series including *S*, Edwards' 05 (activated), *O R* (activated) with *Bact. aërogenes* and *Bact. acidi lactici* as checks was run through fermentation-tubes from two sources of meat, and Edwards' 06, *R*, Edwards'

[1] *Centralbl. f. Bakt.*, 1905–6, 37, p. 338.

05 (activated), and *O* from another source of meat. Where gas was formed, the titration with N/10 NaOH and phenolphthalein was done as soon as the maximum gas formation was reached, before the alkali growth in the bulb could change the closed arm reaction. When there was no gas formation, the reaction was tested at the end of two weeks. No elaborate analysis of the series will be made, as the variations with the different meats were well marked, and by judicious selection the organism could be arranged in almost any classification one chose, except, of course, that the non-fermenters *D* and *Z*, and the inactive *R* and Edwards' 05, fell into a class by themselves on account of the absence of acid.

The fluids from the bulb and closed arm were titrated separately, the latter being drawn off through a curved pipette without allowing it to flow into the bulb. The greatest amount of N/10 NaOH required to neutralize 5 c.c. was 2.5 c.c. and most of the reactions fell between 0.8 c.c. and 2.0 c.c.

In Bertarelli's paper the limits given were wide enough for each group to allow of plenty of doubtful cases at the borders. In general the acid formation is roughly in proportion to the relative amount of H in the formula H/CO_2.

Pathological histology.—Hardened sections from Dr. Marvin's case showed an intact, but somewhat swollen, mucous membrane, beneath which lay new-formed granulation tissue, with a good deal of fully developed connective tissue. The usual text-book description of rhinoscleroma applied to the structure of the mass. In sections stained with eosin and methylene blue, with Giemsa, and to a less extent with hematoxylin and eosin, numerous short, round-ended bacilli were seen, singly and in pairs, both in the "Mikulicz cells" and in lymph spaces. These bacilli were apparently for the most part capsulated. Gram's stain in *alcohol*-hardened tissues showed no organisms, while in the sections hardened in Zenker's, the organisms were visible but not unduly conspicuous.

The stress laid on the presence of the bacteria in the lesions as an indication of the etiological relations to the disease led to the study of sections of other nasal hypertrophies, not rhinoscleroma.

In one case of ordinary *nasal polyp*, somewhat inflamed, eosin-methylene blue showed bacilli and streptococci in the depths of the lesion under a nearly intact mucosa. Four other non-inflamed polypi showed no organisms. In two cases of marked *turbinate hypertrophy*, obtained through the courtesy of Dr. J. M. Ingersoll and Dr. W. T. Chamberlin, organisms of the *B M C* group were obtained, in one case from the surface, in the other from both surface and depths, and in the latter case capsulated diplo-bacilli were found throughout the tissue in sections. The organism in the latter case is that referred to as *O*. In the other case, denoted as *S*, no organisms were found in the sections.

DISCUSSION OF RESULTS.

The conclusions drawn from study of the literature and personal work fall under two heads, the relation of organisms of the *B M C* type to rhinoscleroma, and the relation of apparently non-fermenting organisms of this group to the fermenting varieties.

1. In rhinoscleroma we have a disease with lesions of the infectious

granuloma type, covered with intact mucous membrane, and containing, in special cells and in spaces, bacilli in pairs, with capsules. These bacilli show a variable reaction to Gram according to the hardening method. Cultures from the inside of the tumor-like masses have given growths of diplo-bacilli which had the essential characteristics of the *B M C* group, and were further similar in their pale transparent growth on slant agar. Important differences have, however, been noted by different authors in the fermentation activities. Some of the cultures have been pathogenic, others not so. Transmission of the disease in human beings by means of cultures of the organism has been uniformly unsuccessful, and only one or two successful animal experiments have been reported, none of which appear conclusive.

The endemic quality of the disease, the apparent absence of transmission by contact, in spite of the numbers of organisms in the nasal exudate, and the sporadic type of the disease outside its narrow endemic limits are all against the rhinoscleroma bacillus as a causative factor.

Furthermore, the organisms of this group are well known in disease, but in no case that I have been able to find have there been any lesions of a *productive* type, the only granulation tissue being that which fills up defects after a loss of tissue. General bacteremias, even hemorrhagic in type, are described, but aside from these the typical infection with *B M C* is a pyogenic one, whether in open or closed cavities. This holds good throughout, in the genito-urinary system, the gastro-intestinal tract and peritoneum, and also in the upper and lower respiratory passages, as noted by many authors, in antrum disease, pneumonia, and so forth. In these affections there is always more or less loss of mucosa. Accordingly, if rhinoscleroma *is* caused by an organism of this group, it is in every way a marked exception to other lesions due to the mucosus capsulatus series.

From the pure bacteriological standpoint, authors are not in agreement as to essential details of identification, and even in the present single case several varieties have been found. It seems more easily conceivable that several varieties of organisms living

saprophytically in the nasal cavity should have been taken into the tissues mechanically, than that the lesion is due to the joint effects of several members of one group, especially when the disease has as definite an entity as has rhinoscleroma.

Numerous observers have noted organisms of the rhinoscleroma type in lesions bearing no relation to the disease, and in normal cases. In this paper, *O*, which closely resembles *A*, came from ordinary turbinate hypertrophy, and *S*, resembling *Bact. aërogenes*, from another similar case.

This leaves, so far as I can see, all of Koch's laws unfulfilled, as while organisms of the same general type are found in all cases, they are multiple or non-identical, they do not reproduce the disease when inoculated, and accordingly cannot be obtained from experimental lesions. The weight of the argument based on the finding of the organism in the depths of the tissues seems to me to be seriously affected by my finding organisms in the hardened tissues in a case of turbinate hypertrophy, and an inflamed nasal polyp.

In fine, the evidence obtained from my own work and that of others leads me to believe that the rhinoscleroma bacillus and the disease of rhinoscleroma have no etiological relation, the presence of the bacteria in the cells being due to secondary invasion or ingestion rather than to infection.

2. On the basis of my classification in the former paper, the *B M C* group was divided into three divisions, *Bact. aërogenes*, forming gas in all sugars, *Bact. pneumonicum*, forming gas in dextrose and saccharose, and *Bact. acidi lactici*,* forming gas in dextrose and lactose. The suggestion was made that *Bact. aërogenes*, rather than *Bact. pneumonicum* was the prototype of the group, the others having lost through adaptation, the power to ferment one or the other sugar. It was also stated to be the opinion of the author that the non-fermenting varieties of this group were degenerated or modified fermenters, rather than distinct entities. This was based

* The organism spoken of in this and the preceding papers under the name of *Bact. acidi lactici* differs from the original *Bact. acidi lactici* of Hueppe, in that it forms no spores, but all the cultures I have been able to obtain under this name, whether from Kràl or elsewhere have been alike in this characteristic.

If spores could be demonstrated in the cultures, this finding would at once put the organism out of the *BMC* group, and the name for the third division would have to be changed.

partly on analogy, and partly on the revivification of some old laboratory cultures from Kràl, and the absence of any fermentation in certain cultures obtained from Baltimore with a history of fermentation.

Since the appearance of this paper, Edwards[1] has noted in three cases organisms of this group as etiological factors in acute infections. These organisms, while of the *B M C* group in all other ways, did not ferment, a fact upon which Edwards based the theory that there was a distinct variety of *B M C* which was pathogenic and non-fermenting, and which, on account of its pathogenicity, deserved a position of its own. This organism was given the name of *Bact. mucogenum*, Edwards.

No statement is made as to the source of the media used by Edwards, or the length of time of the fermentation, but it is apparent from the present article that this makes a great difference, as my organisms were extremely weak fermenters in the ordinary Liebig extract broth, and even under most favorable circumstances showed extremely delayed reactions. Two cultures of Edwards obtained a year apart, from Baltimore, were found to ferment after several weeks in favorable meat extracted broth, while in ordinary broth no fermentation took place. This was also true with an organism in my own series. In fact, taking my own series and Edwards organism together, we have organisms which form:

1. Cloud but no gas in *D* and *S*, no cloud in *L*.
2. Gas in *D*, aerobically or in closed arm, no cloud in *L* or *S*.
3. Gas in *D* readily aerobically, very closely in closed arm, no cloud in *L* or *S*.
4. Gas in *D*, *L*, and *S*, with long-delayed reaction, especially noteworthy in *S*.
5. Gas after several weeks in *D*, *L*, and *S*, after which they fall into a class between 4 and 6 in activity.
6. Gas readily in all three sugars.

It thus appears that one may find all grades of activity, and that activity may be restored to an organism which has lost it, either by passage through animals, or by long-continued culture in special media, though not in all cases; furthermore that many organisms, of this group at least, may be set aside as non-fermenters, either from the use of unsuitable media, or insufficient duration of growth.

[1] *Jour. Inject. Dis.*, 1905. 2. p. 431.

In this connection it is of interest to note that organisms which lack power of gas-formation in lactose only, or saccharose only, have in my experience retained their characteristics unchanged, the organisms described in the former paper under those heads being unchanged after three years. All the organisms reactivated in the present series took on a modified aërogenes type.

All this brings up the complicated subject of classification. I notice that in a recent work on milk, it is often stated that the *Bact. acidi lactici* is a variety of *Bact. aërogenes*, and on that basis there is no reason for placing the Friedländer organism, which differs only in the sugar unaffected, in a class by itself.

TABLE 1.

PERIOD IN DAYS BETWEEN INOCULATION AND GAS FORMATION.

NAME	1905 March 8	Apr. 16	May 10	May 11	1906 Jan. 22	Feb. 9	Feb. 28	Apr. 10	Apr. 24
A.....	D+2		(Dextrose	+ 4					
	L o		agar)	o } 32					
	S o		6	o }					
D.....	D	o }	▫	o }	o }		o }		
	L	o } 23		o } 32	o } 42		o } 17		
	S	o }		o }	o }	o 21	o }		
E.....	D+2	+	+	+ 5	+ 2	+ 3			
	L+2	+ 5		+ { 5 12	+ 7				
	S o	+ 13		+ 16	+ 19	+10			
G.....	D+2	+ 2	+	+					
	L+8	+ 2		+ 7					
	S+2	+ 11		+ 20					
M.....	D+2	+	+	+ 4					
	L+2	+ 2		+ { 5 7					
	S+3	+ 10		o 32					
X.....	D	+	+	+ 2	+ 1	+ 3			
	L	+ 8		+ 6	+ 5				
	S	+ 8		o + } 22 + }	+ 19	+17			
Z.....	D	o }	o	o }	o }		o }		
	L	o } 25		▫ } 32	o } 42	o 27	o } 17		
	S	o }		o }	o }		o }		
R.....	D	o }	o	o }	+ 13	+ 3	+ 2	+ 3	+ 2
	L	o } 20		o } 32	+ 22		+ 3	+ 6	+ 6
	S	o }		o }	+ 19		+ 3	+ 3	+ 2
Edwards' 05..	D				+ 21	+ 2	+ 2	+ 3	+ 1
	L				+ 22		+ 7	+ 4	+ 6
	S				o 42		+ 3	+ 4	+ 6
S.....	D						+ 1		+ 1
	L						+ 1		+ 1
	S						+ 1		+ 1
O.....	D							+11	+ 6
	L							o	o } 10
	S							o	o }
Edwards' 06..	D							+12	
	L							+14	
	S							o 23	
Edwards' 05..	(repeat)								
	D							+12	
	L							+14	
	S							o 14	

TABLE 2.

FERMENTATIONS.

Name	Sugar	Cloud Appeared in	Gas Appeared in (Average)	Total Gas	H/CO_2	Reaction of Stem
A	D	1 day	2–4 days	0.50	1/1	+
	L	No	No gas	....	...	−
	S	"	" "	....	...	−
D	D	1 day	None in 42 days	....	...	±
	L	No	" " 42 "	....	...	±
	S	7 days	" " 42 "	....	...	±
E	D	1–2 day	3 days	0.34	2/1	+
	L	3 days	6 "	0.33	3/1	+
	S	10 "	15 "	0.33	2/1	+
G	D	1 day	2 "	0.33	2/1	+
	L	2 days	6 "	0.25	5/3	+
	S	10 "	11 "	0.45	3/1	+
M	D	2 "	3 "	0.25	2/1	+
	L	3 "	5 "	0.33	2/1	+
	S	8+"	10+ "	0.25	2/1	+
O	D	5 "	8 "	0.50	2/1	+
	L	No	No gas	0.00	...	±
	S	Slight 8 days	" "	0.00	...	±
R inactive	D	12 days	13+days	0.33	2/1	+
	L	19 "	20 "	0.33	3/1	+
	S	17 "	21 "	0.20	4/1	+
R active	D	1 day	2–3 "	0.50	2/1	+
	L	2 days	2–5 "	0.50	2/1	+
	S	2 "	3 "	0.60	3/1	+
S	D	1 day	1 day	0.45	3/1	+
	L	1 "	1 "	0.40	3/1	+
	S	1 "	1 "	0.40	2/1	+
X	D	1 "	2 days	0.27	2/1	+
	L	4 days	7 "	0.33	2/1	+
	S		17+ "	0.40	3/2	+
Z	D	1 day	None in 42 days	0.00	...	±
	L	No	" " 42 "	0.00	...	±
	S	3–6 day	42 "	0.00	...	±
Edwards' 05, inactive	D	9 days	21+days	?	?	+
	L	21 "	22+ "	0.33	2/1	+
	S	22 "	42+ "	0.00	...	±
Edwards' 05, active	D	1 day	2 "	0.40	2/1	+
	L	3 days	6 "	0.40	2/1	+
	S	2 "	5 "	0.50	3/1	+
Edwards' 06	D	11 "	12 "	0.60	?	+
	L	13 "	14 "	0.50	?	+
	S	11 "	None in 23 days	0.00	?	±
Bact. aërogenes (stock)	D	1 day	1 day	0.8	?	+
	L	1 "	1 "	0.6	?	+
	S	1 "	1 "	0.9	?	+

TABLE 3.

MILK REACTIONS.

Name	Acid Reaction in	Coagulation in	Alkaline Reaction in	Permanently Amphoteric
A	.....	.....	2–5 days	
B	.....	.....	2–5 "	
C	2 days			
D	.....	.....	.....	4 weeks
E	1–2 days			
G	1–2 "			
M	1–2 "			
O	.....	.....	2 days	
R inactive	.....	.....	6–10 "	
R active	1–2 days			
S	1 day	1–2 days		
X	1–3 days			
Z	.....	.....	.....	4 weeks
Edwards' 05 inactive	.....	.....	5–12 days	
Edwards' 05 active	1–2 days			
Edwards' 06	Slight 2 days			
Bact. aërogenes (stock)	1 day	1–2 days		

CONCLUSIONS.

1. The so-called rhinoscleroma bacillus has no etiological relation to the disease of rhinoscleroma, but is rather a secondary invader.

2. The organisms found in the nose and nasal growths in rhinoscleroma are different in different cases, though of the same general group.

3. Organisms with the power to break up sugars with gas formation may lose such power, in whole or in part, through modifications in environment.

4. Organisms of this group which show no fermentative power are probably degenerated rather than definite entities, and can in many cases be reactivated to their original type.

5. *Bact. aërogenes* is the prototype of the group, and by analogy with *B. coli communis* and *communior*, the other members may perhaps be considered as varieties of it.

6. Until *Bact. pneumonicum* or *Bact. acidi lactici* is activated into *Bact. aërogenes*, the classification formerly brought forward is apparently justified.

SPONTANEOUS PHAGOCYTOSIS OF FUSIFORM BACILLI AND INFLUENZA BACILLI.*

RUTH TUNNICLIFF AND DAVID J. DAVIS.

(From the Memorial Institute for Infectious Diseases, Chicago.)

IT is important to investigate to what extent phagocytosis is dependent upon the presence of opsonins. Are opsonins essential in all cases where micro-organisms are ingested by leucocytes, or may the process occur in some instances in the absence of serum? Again, we may ask whether phagocytosis may not go on to a certain degree without opsonin and the process merely be accelerated by its presence, in a way analogous to the action of a catalytic agent upon chemical reactions. The following observations appear to have a direct bearing upon these points.

PHAGOCYTOSIS OF FUSIFORM BACILLI

(R. TUNNICLIFF.)

The fusiform bacilli employed in the experiments are anaerobic organisms isolated from the gums of healthy mouths. The bacilli, first isolated on ascites agar, were later transplanted on Loeffler's blood serum for several generations because of the possibility of the ascites fluid containing opsonin, which might sensitize the bacilli and thus interfere with the experiment. The human leucocytes used were washed at least five times in normal salt solution in order to remove the serum.

Phagocytosis of both living and dead bacilli in the presence of normal human serum and of salt solution was studied, and the results are given in Table 1.

TABLE 1.

PHAGOCYTOSIS OF FUSIFORM BACILLI.

BACTERIAL SUSPENSION 2 VOLUMES SERUM OR NACL SOLUTION 1 VOLUME WASHED HUMAN CORPUSCLES IN NACL SOLUTION 1 VOLUME	PHAGOCYTOSIS (AVERAGE IN 20 LEUCOCYTES)					
	10 min.	20 min.	35 min.	1 hour	3 hrs.	5 hrs.
Bacteria+serum+washed corpuscles	5.9	7.2	9.9	9.8	9.0	11.0
Bacteria+NaCl sol.+washed corpuscles	7.0	6.5	...	11.0	16.0	25.0
Dead bacteria+serum+washed corpuscles	6.9	10.0	...	9.8	9.0	9.0
Dead bacteria+NaCl sol.+washed corpuscles	3.4	3.4	3.8	6.0	7.0	11.0

* Received for publication September 10, 1906.

From this table the following conclusions may be drawn:

1. Phagocytosis is as active in the presence of normal salt solution as in the presence of serum.

2. Phagocytosis increases in the presence of serum during the first 35 minutes, and remains about stationary after that time.

3. Phagocytosis in the presence of normal salt solution increases both up to and after the first hour, so that at the end of five hours the number of bacteria taken up is more than double the number taken up in the presence of serum during the same time.

4. Dead bacilli are taken up by the leucocytes to about the same extent as the living organisms, showing that the increase in the number of bacilli in the leucocytes is not due wholly to multiplication of the bacilli inside the cells but largely to a true phagocytosis.

In order to ascertain the influence of higher percentages of salt on phagocytosis salt solutions 1.3 per cent, 1.7 per cent, and 2.5 per cent in strength were employed as well as the physiological solution. No difference in the number of bacilli ingested was observed until the strength of the solution was 2.5 per cent, when the number of bacilli taken up was reduced more than one-half.

When the leucocytes were heated for 10 minutes at 50° C. no phagocytosis occurred either in the presence of serum or of salt solution.

The leucocytes and the serum of rabbits and guinea-pigs were also used with the result that phagocytosis of the fusiform bacilli was the same, whether salt solution or serum was employed.

PHAGOCYTOSIS OF INFUENZA BACILLI.

(D. J. DAVIS.)

Pfeiffer noted the occasional occurrence of these bacilli inside the leucocytes in the sputum of influenza patients, more particularly in the latter part of the attack. In the sputum of other infections, such as pertussis, in which similar bacilli are found, it is only occasionally that they are encountered inside the pus cells. When the bacilli in moderate quantities are injected into the peritoneal cavity of animals they are taken up in large numbers by polynuclear leucocytes, but if very large amounts are injected the exudate contains few leucocytes and the animal dies in 12 to 20 hours. The few

leucocytes which may be present under these conditions readily take up the bacilli. *In vivo*, therefore, phagocytosis appears to be an important process in the destruction of influenza bacilli.

In the test-tube strains isolated from measles, whooping-cough, tonsilitis, pharyngitis, varicella, influenza, and conjunctivitis, all were found to be readily taken up by human leucocytes in defibrinated blood in the course of 20 minutes or less. If washed leucocytes are used the bacilli are also taken up but not so rapidly as when serum is present. When thick suspensions of bacilli are used the leucocytes in both cases are crowded with them in from 20 to 30 minutes. If dilute suspensions are used a distinct difference in phagocytic activity between the washed and unwashed leucocytes in favor of the latter is seen in 20 minutes, but after from four to eight hours this difference disappears, the leucocytes being now so filled with the bacilli that their number cannot be accurately determined by count. This phagocytosis in salt solutions which may be referred to as spontaneous phagocytosis cannot be satisfactorily explained by insufficient washing of the corpuscles. In some of the experiments the corpuscles were washed seven times in liberal quantities of salt solution. This amount of dilution and washing is quite sufficient completely to inhibit phagocytosis of staphylococci and meningococci. In several of the experiments staphylococci and meningococci were used as controls, and in no instance were they taken up even after several hours. Again spontaneous phagocytosis cannot be explained by long-continued cultivation on artificial media; all strains isolated from various sources without exception showed spontaneous phagocytosis, though some had been isolated from throat lesions only a few days while others had been under cultivation for five months. The possibility of explaining the large number of bacilli in the leucocytes after a few hours, by growth having occurred inside the leucocyte from an occasional bacillus, need not be considered, because the same result is obtained with bacilli killed by heating to 46° C. for 30 minutes. That the bacilli do not become sensitized by growing on blood media which they require was shown by using washed corpuscles in the media and also by heating the blood to 95° C. for one hour before adding it to the media. Lastly spontaneous phagocytosis is not merely the adhesion of the

bacilli to the surface of the leucocyte, for its appearance is identical with that of phagocytosis occurring in serum, and mere adhesion of bacteria to a leucocyte is manifested by a marginal arrangement of the organisms.

Wright observed spontaneous phagocytosis of tubercle bacilli in certain concentrations of salt solutions. The greatest spontaneous phagocytosis occurred in a 0.6 per cent solution; this progressively decreased and practically disappeared in a 1.3 per cent solution. Varying concentrations of salt solution, at least within the above limits, have no effect upon the phagocytosis of influenza bacilli. This is shown in Table 2. In this experiment a thin suspension of the

TABLE 2.

SHOWING EFFECT OF VARYING CONCENTRATIONS OF NaCl UFON PHAGOCYTOSIS OF INFLUENZA BACILLI BY NORMAL HUMAN CORPUSCLES WASHED FOUR TIMES.

CONCENTRATION OF NaCl SOLUTION	PHAGOCYTOSIS		
	20 min.	2 hours	7 hours
0.6 per cent	0.5	3.1	20+
0.7	0.3	2.4	20+
0.8	0.9	4.8	20+
0.85	0.3	4.0	20+
0.9	0.4	8.5	20+
1.0	0.1	0.9	20+
1.1	0.15	1.2	20+
1.2	0.1	8.0	20+
1.3	0.2	3.8	20+
1.4	0.3	3.9	20+

bacilli was used, otherwise phagocytosis would have been so rapid the bacilli could not be counted in 20-minute and two-hour intervals.

GENERAL CONSIDERATIONS.

On the basis of these results we are therefore justified in considering the influenza and fusiform bacilli as organisms for the phagocytosis of which serum is not necessary. In this respect they may be classed with such inert bodies as carmin particles which, as is well known, are taken up by washed leucocytes.

From the facts brought out in our experiments it would seem that opsonins are not the only factor which controls the ingestion of bodies by leucocytes.

In connection with the facts here stated concerning influenza and fusiform bacilli may be mentioned certain other, closely related phenomena, not infrequently observed, which seem difficult to

explain on the basis solely of the opsonic theory. In suspensions of leucocytes, thoroughly washed, and bacteria (staphylococci, meningococci, colon bacilli, etc.) one finds occasionally a leucocyte containing a large number of organisms, whereas most of the cells under apparently the same conditions do not contain any bacteria; and one may nearly always find that a few bacteria have been taken up. The opsonic index is rarely ever zero under these conditions. Again, in the meningeal fluid in cases of meningitis it is very common to find here and there a leucocyte with many meningococci, while most of the cells do not contain the cocci, though identical morphologically with those which do. In these cases the fluid contains very small amounts of opsonin compared with serum as may be shown by test-tube experiments. One cannot attribute such phenomena to clumping of the organisms, for this is not apparent; and it does not appear to be due to an intraphagocytic multiplication of the bacteria. The facts, therefore, appear to indicate that in this case the leucocytes do not, at least not in all cases, play merely a passive rôle in the process of phagocytosis, taking up anything that may be prepared for them by the serum, but more probably exercise a selective function which is comparable to the ingestion of food by the ameba.

It is to be noted that in the case of influenza bacilli the serum accelerates the process of phagocytosis, which goes on to a considerable extent, however, with washed leucocytes. One might explain the phagocytosis in the latter instance by saying that all of the serum was not removed by washing and that the bacilli are sensitized by minute quantities of opsonin, whereas other organisms, such as the staphylococcus, are not appreciably affected by such small amounts. It would be difficult to prove or disprove directly this point, inasmuch as it is impossible theoretically to remove all the serum from the leucocytes by the method of washing in salt solution. However, after washing seven times, as was done in some of our experiments, the quantity of serum left must be very small indeed. Hence it does not appear reasonable that the minute traces of serum which may remain play any detectable rôle in the so-called spontaneous phagocytosis.

There seems to be an analogy, and it can be considered merely an

analogy, between the auxiliary action of serum in phagocytosis of influenza organisms and the catalytic action of ferments in chemical reactions whereby the process which normally goes on very slowly is accelerated by the presence of the catalyser. In the case of fusiform bacilli this auxiliary action is not so apparent, though, as may be seen from Table 1, there is a distinct suggestion of it when dead bacilli are used.

One may assume that the bacteria, taken up by spontaneous phagocytosis, in the natural state may be in such a condition that they do not require opsonification. One may say that they are in an opsonized condition naturally. We may suggest further that the bacterial cell itself may produce opsonin that enables leucocytes to ingest it, thus being in the presence of leucocytes its own destroyer. It is as rational to assume the production of opsonin by bacterial cells as by other cells or the production of cell receptors by bacteria. The objection to this idea, however, is the fact that bacteria heated to a point (60° C.) which appears to destroy many opsonins as we now know them, are still taken up by washed leucocytes. This would consequently rule out the possibility of the origin in the bacterial cell of an opsonin having the properties of most normal serum opsonins. We may state then that if certain bacteria are opsonized naturally, it must be due to a body with properties different from that of ordinary normal opsonins in that it is more highly resistant to heat. This is not impossible, for we are acquainted with immune opsonins which are much more resistant to heat than normal opsoonins and which probably vary considerably in this respect. We might even consider the suggestion that immune opsonins in the animal body originate from the bacterial cell.

There still remains the possibility that the natural physical state of certain bacteria, such as the surface properties, favor the process of phagocytosis. This may be applied not only to bacteria but to inert bodies like carmin particles. Indeed, it is quite probable that ultimately the whole process of phagocytosis and opsonification will be found to depend upon such factors; but we should be extremely cautious in assuming that we have here to deal only with such comparatively simple phenomena, as variations in surface tension or electrical charge. For, granting that such variations occur, we know

nothing as yet of a host of other processes which are undoubtedly set up at the same time in the delicately balanced protoplasmic system and which may either counteract or reinforce the original disturbance. The facts at present seem to indicate that we must occupy an intermediate position and consider both the leucocyte and the state of the bacterial cell as playing an important and quite independent part in the process, and that it is difficult to interpret all the facts concerning phagocytosis on the basis of the opsonic theory, according to our present conception of the term opsonin.

HEMOPHILIC BACILLI—THEIR MORPHOLOGY AND RELATION TO RESPIRATORY PIGMENTS.*†

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INTRODUCTION.

PFEIFFER in his classical work[1] upon influenza in 1893 gave a very clear and comprehensive report on the biology of the bacillus that he found associated with the disease which at that time was pandemic. He also found in a few cases of broncho-pneumonia a bacillus somewhat larger than the influenza bacillus and with a marked tendency to form threads; in other respects it closely resembled the influenza bacillus, and he therefore called it the pseudo-influenza bacillus. The most characteristic property of these bacilli is that they require for their growth the presence of hemoglobin and therefore have been frequently called, especially by the French, hemophilic bacteria. Since Pfeiffer's work organisms of a similar character have been found in the throats in a large variety of diseases, especially those of an infectious character, and also occasionally in normal throats.[2] By many they have been considered identical with the influenza bacillus, and the cases in which they were found have been looked upon as double infections, the influenza bacilli being secondary to some other invader. By some observers certain hemophilic bacteria are regarded as the cause of whooping-cough because of their occurrence in the sputum in this disease, but definite proof of their etiologic rôle is still lacking.

It is the purpose of this paper to compare hemophilic bacilli obtained from various clinical conditions with one another, especially from the standpoint of morphology and cultural reactions, not only toward hemoglobin and its modifications but also toward other respiratory proteids which occur in lower animals.

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[1] *Ztschr. f. Hyg.*, 1893, 13, p. 357.

[2] Davis, "The Bacteriology of Whooping-Cough," *Jour. Infect. Dis.*, 1906, 3, p. 1.

Hemophilic bacilli were obtained chiefly from the sputum, from a variety of conditions including measles, whooping-cough, varicella, bronchitis, cerebro-spinal meningitis, tonsilitis, clinical influenza (grippe), otitis media following measles, and normal throats. Over 100 strains of the bacilli were isolated in pure culture and their morphology and cultural properties noted. The means of isolation was the blood-agar plate. The sputum is washed in water or broth and tubes of melted agar, to which a few drops of blood (preferably pigeon) has been added, are inoculated with washed sputum, and poured into sterile plates. After 24 and 48 hours the plates are carefully examined with the naked eye and also with a hand lens or low-power microscope for the minute dew-drop-like colonies characteristic of hemophilic bacteria, which now are transferred to blood-agar tubes and studied further.

MORPHOLOGIC CHARACTERISTICS.

In smears made from the sputum directly the bacilli are always single with practically no tendency to form threads; neither are they arranged end-to-end in chain formation. In some particles of sputum they are much more numerous than in others but a characteristic grouping does not occur. The arrangement of the bacilli in longitudinal rows or like a school of fish, frequently observed in smears of sputum, is purely an artefact due to the smearing, and not, as some have thought, a characteristic and natural distribution of the bacilli in the mucus. The bacilli are small rods with rounded ends about two or three times as long as broad. They are non-motile, do not have a capsule, do not form spores and do not take Gram's stain. With methylene blue they stain more deeply at the ends; with carbol-fuchsin they stain very distinctly and quite uniformly. In cultures there is more variability in the morphology of the bacilli. Many strains possess a remarkable tendency to form threads though showing none of this in the sputum. All the strains in cultures show it to some extent, but vary considerably in this respect. This tendency often shows itself in the first generation on the plates and in the first 24 hours. Not only do strains, isolated from various conditions, show marked variation, but the same strain at different times may vary. On the whole it may be said that the

more rapidly and the more luxuriantly the bacilli grow the less is this tendency, while the more unfavorable the media the more thread-forms appear; but if these latter are again grown under more favorable conditions they revert to their original form. As a rule, too, the thread-forms are more numerous in a culture a few days old than in a fresh one. The threads are occasionally very long, sometimes crossing the entire field of the microscope, and are often curved in various shapes. They are much wider than the single typical bacillus and usually stain well and quite uniformly.

In view of the fact that these bacilli have such a tendency to produce anomalous forms on ordinary media, and especially under slightly unfavorable conditions, the growth of seven strains was tested upon blood agar containing sodium chloride in varying concentrations. Upon media with a sodium-chloride content of 2 per cent to 3 per cent unusual forms are encountered invariably. In 24 hours at 37° C. only a few peculiar forms are noted; but in 48 hours a great variety of them appears. Swollen forms which assume various shapes are especially numerous. Sometimes the whole bacillus is transformed into a large, round, irregularly staining coccus. Again, one or both ends of the bacillus may be greatly swollen and frequently the ends stain deeply with methylene blue, often resembling very closely certain forms of diphtheria bacilli, the deeply staining parts looking much like granules. While some thread-forms appear they do not occur in great numbers nor to the extent that they do at times on ordinary blood agar. The strains differ from one another considerably in their appearance under these conditions and the same strain may vary considerably at different times.

Hankin and Leumann[1] called attention to the occurrence of degeneration and involution forms of the pest bacillus when grown on NaCl agar and suggested this as a means of separating this organism from other organisms having a similar appearance. Since then it has been shown by several, especially by Matzuschita[2] and Rosenfeld[3] that many other varieties of bacteria display this phenomenon on NaCl agar after 48 hours or later, but that the forms of the pest

[1] *Centralbl. f. Bakt.*, 1897, 22, p. 438.
[2] *Ztschr. f. Hyg.*, 1900, 35, p. 495.
[3] *Centralbl. f. Bakt.*, 1901, 30, p. 641.

bacillus occur in 24 hours and are sufficiently characteristic to be of value in its differentiation. The influenza bacilli, therefore, behave on sodium-chloride media like many other forms of bacteria, but there is nothing characteristic in these changes and they are therefore of no service in distinguishing these bacilli from other forms; neither do the various strains show any characteristic appearances which might indicate differences between them.

CULTURE OF HEMOPHILIC BACILLI.

In the cultivation on hemoglobin agar of numerous strains of hemophilic bacilli from the various clinical conditions already noted no differences were seen. On plates they appear as small, non-hemolyzing, clear, dew-drop-like colonies, as a rule, pin-point in size, and often requiring the aid of a hand lens for detection. By reflected light they appear a delicate, pale blue. Under a low power they are quite homogeneous, circular or oval in shape, have a regular margin and no central nucleus. On blood-agar tubes a delicate growth occurs along the needle track, often difficult to see at first glance. The colonies tend to remain discrete.

Hemoglobin seems to be a necessary substance for the continued growth of the bacteria. Pfeiffer first showed this for the influenza bacillus, tests on many other kinds of media aways giving negative results. Cantani[1] says that he obtained growth of influenza bacilli upon spermatic fluid, and many other substances free from hemoglobin, and thinks that globulin may be the active part of hemoglobin, which induces their growth; but Ghon and Preyss[2] showed that the media he used were not entirely free from hemoglobin or hematin. Fichtner[3] also contends that hemoglobin is not necessary, and thinks there is some substance in the red cells which is essential for multiplication and explains certain symbiotic phenomena in this way. He says he succeded in getting a good growth on agar if sputum, heated to 60–65° C. for several days until sterile, was mixed with it. Notwithstanding these and a few other conflicting results, most observers are agreed that hemoglobin is the essential substance and that no

[1] *Ztschr. f. Hyg.*, 1901, 36, p. 29.

[2] *Centralbl. f. Bakt.*, 1904, Orig., 17, p. 531.

[3] *Ibid.*, Orig., 35, p. 374.

other substance has yet been found to take its place successfully in the cultivation of influenza bacilli.

Pfeiffer used the blood of various animals, including man, rabbit, guinea-pig, pigeon, and fishes, and found pigeon blood to be the most successful of all the bloods tested for this purpose. He thinks this is due to the instability of the pigeon corpuscles which allows the hemoglobin to diffuse more readily into the media. Whether this latter statement is true or not, it does appear to be a fact, as many have determined that pigeon blood is a very suitable medium, and, as a rule, more luxuriant growth is obtained on this than with many other bloods. I have used pigeon blood very largely in the isolation of the bacilli and it seems more reliable than other bloods and especially more so than human blood.

Because of the value of pigeon blood in the cultivation of hemophilic bacilli a convenient method of obtaining it in large quantities in a sterile condition becomes important. In 1902 Czaplewski[1] described a method of making an incision into the large breast muscle of the pigeon with a sharp lance; as the blood oozes from this wound, it is drawn into a small pipette and introduced into melted agar. Another method frequently used is that of pricking a vein in the wing of the bird and allowing the blood to drop into tubes of media or upon agar plates. Evidently these methods are unsuitable when a large amount of pigeon blood is desired and when defibrinated blood or a quantity of serum is wanted. A more convenient and rapid method, certainly less liable to result in contamination, is one which I have used, the details of which are as follows: The feathers are removed on the left side of the pigeon directly underneath the wing, exposing the skin for several square centimeters. The skin is then thoroughly cleansed with 5 per cent carbolic acid and alcohol. The pigeon lying on its right side, an assistant holds up the left wing and extends the left leg. At a point approximately midway between the posterior skin fold of the wing and a fold of the skin found just anterior to the thigh when the bird is in this position, the needle of the Luer syringe is introduced directly into the heart. The needle passes just above the margin of the breast bone and proceeds, approximately vertical to the surface, inward about 1.5 to 2 cm., when the beat of the heart may be distinctly felt against the needle. The heart beat is important and should always be obtained before proceeding further. Advancing the needle a short distance the blood will be seen to enter the syringe. The needle should be sharp so as to enter the heart readily, of medium size, and from 3 to 4 cm. in length. Large needles do not enter the heart readily and are apt to push it aside, while small needles allow the blood to pass too slowly into the syringe, furnishing an opportunity for clotting. By dissecting a dead pigeon one can readily determine the proper position at which to enter the heart.

Four c.c. may be removed without any evil effects. If more is taken the animal usually shows weakness of the legs and other signs, but frequently recovers if 7 or 8 c.c. are withdrawn. The needle is removed cautiously and practically no bleeding occurs

[1] *Centralbl. f. Bakt.*, 1902, 32, p. 667.

at the site of the puncture. The blood is at once transferred to a sterile tube and defibrinated with a wire. This usually takes from two to four minutes. Four c.c. can be withdrawn from the same bird once every week or 10 days and produce no ill effects. I have one pigeon which has been bled from the heart about 25 times in six months and has furnished over 100 c.c. of blood in that time, yet it is as fat and plump as when first bled. After the fibrin is removed the blood can be kept in a capped test-tube in the ice-box for several weeks. For plating about six drops are introduced into melted agar cooled to 43° C., which is then inoculated and poured into the Petri dish. For agar slants one or two drops allowed to run over the surface are amply sufficient. Or a solution of hemoglobin may be obtained by adding distilled water to the blood, thus increasing its volume several times. Four or five drops of this solution suffice to cover the agar slant, or the blood or hemoglobin may be added directly to melted agar, which is then slanted. Such tubes are very convenient, as the influenza colonies are easily seen on the surface, and in transferring an organism to non-hemoglobin media for the purpose of testing its growth there is much less danger of removing hemoglobin with the organisms on the platinum loop.

Careful examination of the propreties of hemoglobin derived from various animals shows that they are not all identical, the hemoglobins varying in chemical composition, in solubility, in crystalline form, and in the quantity of water of crystallization, but apparently all perform the physiological function, of transmitting oxygen. It is therefore important to test the blood of a number of both warm and cold-blooded animals to see if the differences in the hemoglobin have any effect upon the growth of the bacilli. The blood, obtained in sterile condition, was added to plain agar both by smearing on the surface of slants and also by mixing it thoroughly with melted agar and then slanting the tube. The results are given in Table 1.

TABLE 1.

GROWTH OF HEMOPHILIC BACILLI IN MEDIA CONTAINING BLOOD OF VARIOUS ANIMALS.

	Growth of Bacilli	Respiratory Proteid in Blood
Mammals	+	Hemoglobin
Birds	+	"
Perch	+	"
Eel (*Anguilla chrysypa*)	+	"
Dog-fish (*Mustelus canis*)	+	"
Snapping turtle (*Chelydra serpentina*)	+	"
Painted turtle (*Chrysemys picta*)	+	"
Frog	+	"
King crab (*Limulus polyphemus*)	o	Hemocyanin
Lobster (*Homarus americanus*)	o	"
Spider crab (*Libinia dubia*)	o	"
Clam (*Mya arenaria*)	o	"
Phascolosoma Gouldii	o	Hemerythrin
Nereis virens	?	Hemoglobin
Sea-cucumber (*Thyone briareus*)	o	"
Star-fish (*Asterias vulgaris*)	o	"
Sea-urchin (*Arbacia punctulata*)	o	Echinochrom

Inasmuch as hemoglobin belongs to an interesting class of substances known as respiratory proteids, it is important that the growth of influenza bacilli be tested with other compounds of this class, especially hemocyanin, a common proteid in many of the lower animals. This substance apparently performs the same physiological function as hemoglobin but contains copper in large amounts and no iron. The results with the blood of these lower forms are also given in Table 1. Some of the bloods tested were from marine animals* in many of which the salt content is about the same as that of sea-water (3–4 per cent). The blood, however, was diluted to such an extent in the media that the salt content could have no special effect on the growth of the bacilli which grow on media containing as high as 3 per cent sodium chloride.

In Table 1 "mammals" include the following animals: man, dog, rabbit, guinea-pig, ox, sheep, and horse. Among the birds blood from the pigeon and hen were tested. The bloods containing hemocyanin were examined for hemoglobin with a spectroscope so as to exclude the possibility of this substance being present. With the blood of the sea-cucumber (*Thyone briareus*) the spectrum of hemoglobin is distinctly obtained. In its blood are seen large, round, nucleated cells, staining deeply with eosin, which contain the pigment. It is difficult to get the blood in appreciable quantities and sterile. I succeeded twice in getting small quantities in a sterile condition, but in neither case did the influenza bacilli grow in media made with this blood. This is the only instance I have found in which the growth of hemophilic bacilli was not obtained in the presence of hemoglobin. If one considers the echinoderms as being below the annelids in the zoölogical scale, as is commonly done, then the sea-cucumber is the lowest animal in which hemoglobin occurs. From *Nereis*, an annelid in which the hemoglobin is dissolved in the plasma, I was never able to get the blood in an absolutely sterile condition. The bacilli grew upon this blood, but since there were a few other organisms present, symbiosis probably influenced the result. By heating the blood for several days at 65° C it was rendered sterile, and upon this heated blood the bacilli did not grow. After such

* This part of the work was done at the Marine Biological Laboratory at Woods Hole during the summer of 1906.

prolonged heating, however, all the hemoglobin was probably changed to hematin, so that this negative result is of no value.

The body fluid of *Phascolosoma*, another annelid, according to Schwalbe[1] contains a respiratory pigment which is called hemerythrin. It does not give a characteristic spectrum, contains iron, and unites more firmly with oxygen than hemoglobin. This fluid is easily obtained in large amounts in a sterile condition. Many attempts to grow hemophilic bacilli on media to which this fluid was added always gave negative results.

Hemocyanin is readily obtained in a sterile condition from a number of the lower animals. Hemocyanin agar was made containing varying quantities of hemocyanin, both unheated and heated (60° C. for 30 minutes); in no instance did growth of the bacilli occur. When staphylococci were streaked on hemocyanin agar after inoculating with influenza bacilli the latter grew no better than upon plain agar when so streaked (influenza bacilli often grow, at least through a few generations, in conjunction with another organism in the absence of hemoglobin). Hemophylic bacilli do not grow on media containing echinochrom, an iron-containing pigment from the sea-urchin.

From Table 1 we may conclude then, that hemophilic bacilli can use the hemoglobins from a large variety of animals, both warm and cold-blooded, fresh and salt-water forms. The differences between the hemoglobins in their chemical and physical properties do not appear to have any effect upon their growth.

Hemocyanin, hemerythrin, and echinochrom cannot be used by the bacilli even though they appear to have the same function, i. e., are oxygen-carriers, as hemoglobin.

Cantani and Fichtner, as above stated, maintain that certain substances in the cells other than hemoglobin are suitable for the growth of influenza bacilli. In animals, like Limulus, whose blood contains hemocyanin but no hemoglobin, there is an abundance of white cells, but these appear to be quite unsuitable for the growth of the bacilli; but when a little hemoglobin is added growth occurs at once. It does not appear that there is any substance in the blood of animals, containing hemocyanin, which prevents growth when

[1] *Arch. f. mikroskop. Anat.*, 1896, 5, p. 248.

hemoglobin is present, and it consequently does not seem probable, as Cantani contends, that cell substances, such as forms of lecithin, cholesterin, or other bodies, are able to induce growth of the bacilli.

In the cultivation of hemophilic bacilli upon various special media not containing hemoglobin I have had no success. Yolk of egg smeared on or mixed with agar gives negative results. The hematogen, therefore, of Bunge, an iron compound, supposed to be the prosubstance of hemoglobin in the egg, does not appear to be useful for the growth of these organisms. Sputum, filtered through clay or rendered sterile by heating at 65° for several days and mixed with agar, likewise gave negative results. Bile obtained with care to avoid the presence of hemoglobin gave negative results.

In order to determine, if possible, whether the nutrient property of hemoglobin is dependent upon its power of giving up oxygen readily, chemical substances which give up oxygen easily were added in small quantities to the media, but growth of the bacilli did not take place on media containing sodium nitrate, sodium nitrite, potassium chlorate, collodial platinum, hydrogen peroxide, and collodial platinum plus hydrogen peroxide. When collodial platinum and hydrogen peroxide together are added to melted agar, the medium in 24 hours is filled with small bubbles of liberated oxygen. Under these conditions, however, no growth was obtained. Iron salts of various kinds in small amounts were added to media, but in no instance was growth of influenza bacilli detectable; this agrees with the results obtained by Pfeiffer and others.

Hematin, the iron-containing moiety of hemoglobin, according to several observers will not support the growth of influenza bacilli; according to Ghon and Preyss[1] it will do so if another organism (staphylococcus) is present. Since they will grow for several generations when mixed with another organism on media which is spectroscopically free from hematin, the latter observation is of little significance. Pure hematin dissolved in dilute alkali and added to media in varying proportions gave uniformly negative results. The experiment was properly controlled to rule out any possible inhibiting effect of the alkali upon the growth of the bacilli.

[1] *Centralbl. f. Bakt.*, 1904, Orig., 17, p. 531.

Experiments were made to determine the effect of heated hemoglobin on the growth of hemophilic bacilli. At a temperature of about 65° C., or slightly less, a solution of hemoglobin is coagulated. In media containing solutions of hemoglobin heated for 30 minutes or one hour at this temperature the influenza bacilli will grow. If the heated solution is smeared on the surface of agar slants, the influenza colonies cluster about the clumps of coagulated hemoglobin. The bacilli will even grow in the presence of hemoglobin heated to boiling for a few minutes. But if the solution is heated to 65° C. for several days, or in an autoclave at 110° C. for 30 to 60 minutes, then the bacilli no longer can use it. Now at a temperature of 60° C. or less oxyhemoglobin is slowly split into its constituents, hematin and an albuminous portion, and in view of this fact the results just noted are readily explained. Heating to 65° C., or even to 100° C., for a short time is not sufficient to split all the oxyhemoglobin. But by heating for a long period at a temperature of 60°–65° C., or at a higher temperature in an autoclave for an hour, the hemoglobin is split entirely into its constituents and then is no longer serviceable for the bacteria. One cannot directly follow the transformation of hemoglobin with a spectroscope because coagulated hemoglobin does not give a spectrum.* If, to an aqueous solution of oxyhemoglobin, a small amount of sodium carbonate is added and then heated to 55° C., the solution in a short time becomes deep-brown in color but no coagulation occurs, even when heated to boiling. The spectroscope shows that the hemoglobin spectrum is replaced by that of hematin. In such a solution influenza organisms will not grow, showing again that alkaline hematin cannot be used by these bacteria.

We may therefore conclude from these experiments that hemophilic bacilli can use hemoglobin and coagulated hemoglobin in their development, but as the hemoglobin is split into its constituents by heating it loses its power to support their growth.

* Since the spectroscopic test cannot be applied to coagulated hemoglobin the question may be raised whether or not coagulation of hemoglobin is not merely a decomposition into hematin and a globulin since the splitting of hemoglobin into hematin and an albuminous portion begins below the temperature of coagulation. The fact that influenza bacilli cannot grow on hematin and can grow well in the presence of coagutaled hemoglobin may be used as evidence that this process of coagulation of hemoglobin is not, at least altogether, a transformation of hemoglobin into hematin and a globulin.

AMOUNT OF HEMOGLOBIN NECESSARY FOR THE GROWTH OF HEMOPHILIC BACILLI.

In order, if possible, to throw some light upon the rôle that hemoglobin plays in the development of hemophilic bacteria quantitative experiments were made to determine how much hemoglobin in solid media is necessary.

Human blood was diluted in distilled water 1,000 times, and this solution was added to measured quantities of melted agar at a temperature of 42° to 45° C. In this way various dilutions of the blood were made and the growth of the bacilli tested with them. Assuming that hemoglobin is the essential element in the blood for the growth of these organisms and knowing the amount of hemoglobin in blood we can easily calculate the amount of hemoglobin in the various dilutions. Preyer[1] has determined that in human blood hemoglobin makes up approximately 14 per cent or one-seventh of the weight of the blood. Therefore by multiplying the dilution of blood by seven we get approximately* the dilution of the hemoglobin.

In transferring from the stock culture it is necessary to keep in mind that some of the hemoglobin may adhere to the bacteria and the needle; in order to eliminate this source of error transfers must be made through several generations. A typical hemophilic organism isolated from a case of whooping-cough was transferred from a diffuse blood-agar culture, care being taken just to touch the surface of the growth so as to transfer as little as possible of the hemoglobin. In this first series growth occurred at a dilution of one part of hemoglobin to 180,000 parts of media. The controls on plain agar showed no growth. Transfers were now made from the first series of tubes to corresponding dilutions in a second series, and so on through five generations. In the second generation the highest dilution at which growth occurred was 1:60,000; in the third generation, 1:90,000; in the fourth, 1:180,000; and in the fifth, 1:60,000. Careful controls were made on the same media as that to which the hemoglobin had been added. Similar experiments made with pigeon blood gave essentially the same results. This would seem to indicate

[1] *Am. Text Book of Phys.*, 1, p. 38.

* The hemoglobin content of blood from different animals and from the same animal varies somewhat, so that the figures are only approximate. For our purpose, however, it is necessary only to obtain approximate dilutions.

that pigeon blood is no more favorable for the growth of the bacilli than other blood at least in a laked condition and in high dilutions. With dog blood the highest dilution at which growth occurred was 1:90,000.

The hemoglobin solutions in high dilutions deteriorate rapidly, so that it is necessary to use fresh solutions each day. A solution of 1:10,000 in a few days changes its color from a delicate pink to a light yellow, and with such solutions the bacilli grow only at much lower dilutions.

The results show that these bacilli are very sensitive to small amounts of hemoglobin, and this raises the question as to how hemoglobin favors the growth of the organisms. In the extremely high dilutions it does not seem that the hemoglobin can be of much importance as a nutritive factor. It is more probable that it plays the part of a ferment in some essential process, perhaps that of respiration. While the process may in some way be dependent upon the iron, owing to its peculiar combining relationships with oxygen in the hemoglobin molecule, the iron in itself cannot be said to be more essential than any other element, because every compound of iron tested was of no value in cultivation of the bacilli. Oxidases are not the determining factor, for the blood of lower forms contains them, yet without hemoglobin they are of no avail. One naturally associates the function of hemoglobin as an oxygen-carrier with its function of causing hemophilic bacteria to grow. But hemocyanin, a copper compound, apparently performs the same function as an oxygen-carrier in many of the lower animals, but the bacilli cannot use it. The copper in this form does not appear to be fatal to them, since, as we have seen above, they will grow in media containing hemocyanin if hemoglobin is present. Hemerythrin, occurring in *Phascolosoma*, is an iron-containing compound similar to hemoglobin, but it does not permit growth. According to Luerssen[1] chlorophyll, a substance closely related to hemoglobin in some respects but containing no iron, does not favor the growth of influenza bacilli. The facts at present therefore permit us to state that hemophilic bacteria will grow only in the presence of hemoglobin and very minute amounts of this body suffice for this purpose. This

[1] *Centralbl. f. Bakt.*, 1904, 35, p. 437.

suggests that the hemoglobin acts as a catalytic agent rather than as a nutritive substance.

In connection with these facts should be mentioned the favoring influence that growing bacteria of another variety have upon hemophilic organisms. My own experiments indicate that continuous growth of hemophilic bacteria will not take place in the presence of other bacteria, but that they die out after a few transfers have been made. Some observers, especially Neisser,[1] claim to have succeeded in carrying them in mixed cultures through many generations. However this may be, it is certain that other living organisms distinctly favor the growth of hemophilic bacteria, and the most luxuriant growth is obtained by a combination of hemoglobin and the presence of another organism, as is done, for instance, by inoculating the whole surface of a blood-agar tube with influenza bacilli and then making a staphylococcus streak through the center. It would be reasonable to suppose that there is something in common in the way in which these two favoring influences operate, but on this point we can only speculate.

OTHER PROPERTIES OF HEMOPHILIC BACTERIA.

In a number of strains of hemophilic bacteria grown for about one year and transferred every 10 to 14 days, the properties and morphology have remained practically unaltered.

In fluid media they live longer as a rule than in solid media, which is probably due to the effect of drying. On solid media in uncapped tubes I have several times obtained growth after an interval of one month. In sealed glass tubes kept at room temperature in the dark the bacilli grew after three months. In another tube similarly kept and examined after eight months, the bacilli showed no growth upon the inoculation of fresh media.

In distilled water the bacilli gave good growth at the end of 24 hours, scanty growth at the end of 48 hours, and none at all after 72 hours. Dried on a free surface kept sterile the bacilli gave good growth after three hours, slight growth after 24 hours, and none after 48 hours. In human defibrinated blood the bacilli at the end of 10 days were alive and present in abundance. In human serum

[1] *Deutsche med. Wchnschr.*, 1903, 29, p. 462.

no growth was obtained after 24 hours. The organisms, then, are not highly resistant, dying rapidly in water and being quickly killed by drying; in defibrinated blood, however, they find a favorable medium for development and remain alive for a long period.

They grow best at 36°–38° C., and do not grow at room temperature. Growth is first detected at a temperature of about 28° C. A temperature of 42°–43° C. for a few hours is fatal.

The organisms after growing on artificial media containing small amounts of hemoglobin (one drop of blood and frequently less to 5 c.c. of melted agar) for many months do not seem to lose their hemophilic property to any perceptible degree, for they show not the least evidence of multiplication when transferred to non-hemoglobin media. It is possible that by much longer cultivation and on media containing much less hemoglobin they may adapt themselves to plain media, but no evidence for this has thus far been obtained.

CONCLUSIONS.

1. Various strains of hemophilic bacteria isolated from different conditions cannot be differentiated on morphologic grounds.

2. They can utilize in their growth hemoglobins from various warm and cold-blooded animals and also from fresh-water and marine forms.

3. They are not able to utilize other respiratory proteids, e. g., hemocyanin, hemerythrin, echinochrom.

4. An extremely small amount of hemoglobin in the media (1 part in 180,000 parts of media) is sufficient for their development.

5. No satisfactory cultural results have been obtained with any substance other than hemoglobin. They do not grow in the presence of hematin.

THE PATHOGENICITY OF *STREPTOCOCCUS LACTICUS*.*

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IT has been shown by many observers that streptococci frequently occur in milk and Escherich[1] is of the opinion that infants fed on cow's milk have especially large numbers of streptococci in the intestines. These streptococci he found fatal to mice in some instances. Milk streptococci have been found pathogenic by some but not all observers. Beck[2] injected the centrifugal residue of milk into guinea-pigs which then died of peritonitis caused by streptococci. Pure cultures of this streptococcus in mice brought about a fatal general infection. Rubbing on the ear produced reddening of the skin in some instances. Bergey[3] injected streptococci from milk into the skin of the ears of rabbits with the result that slight local lesions developed. The amount injected is not stated. Lammeris and Harrevelt[4] recovered a streptococcus from milk which had been the cause of serious stomach troubles and diarrhea in adults, but found it non-pathogenic for rabbits and guinea-pigs. Park and Holt[5] stated that 40 per cent of all bacteria injected into guinea-pigs proved pathogenic, but whether streptococci were among these is not stated. V. Freudenreich[6] inoculated rabbits with streptococci from milk with negative results. Hölling[7] inoculated 11 mice with as many strains of *Strept. lacticus*. Three of these died. Brüning[8] found streptococci in 93 per cent of Leipzig milk which produced fatal general infections in mice but did not affect guinea-pigs. Savage[9] obtained negative results from milk streptococcus

* Received for publication October 20, 1906.

[1] *Jarhb. f. Kinderheilk.*, 1899, 49, p. 137.

[2] *Deutsche Vierteljahrsschr. f. öffentl. Gesundheitspfl.*, 1901, 32, p. 430

[3] *Am. Med.*, 1901, 1, p. 122..

[4] *Zeitschr. f. Fleisch u. Milch Hyg.*, 1901, 11, p. 114.

[5] "Upon the Results with Different Kinds of Pure and Impure Milk in Infant-feeding in Tenement Houses, etc.," *Archiv. of Pediatrics*, 1903.

[6] *Centralbl. f. Bakt.*, Abth. 2, 1904, 13, p. 281.

[7] *Inaug. Dissert.*, Bonn, 1904.

[8] *Jahrb. f. Kinderheilk.*, 1905, 12, p. 1.

[9] *Jour. Hyg.*, 1906, 6, p. 123.

injections. Müller[1] stated that the speed of coagulation of milk streptococci and pathogenic streptococci differs materially, but that there is no difference in agglutination or hemolysis. Kaiser[2] found streptococci in 76.6 per cent milk after ruling out diplococci and short chains.

It is probably true, then, that streptococci freshly isolated from milk, apart from those originating from mastitis, do not possess high virulence. I have shown[3] that *Strept. lacticus* occurs constantly in milk and since the morphological and cultural characters of this variety of streptococcus on ordinary media are identical with those of *Strept. pyogenes*, it seemed likely that the virulence might be increased by successive passages through animals.

Experiments along this line have been carried on by the writer with rabbits, the rabbit being chosen because of its susceptibility to streptococcus infection.[4] The following plan was pursued. Various cultures of *Strept. lacticus* were "rejuvenated" by passing through three tubes of serum broth, and 2 c.c. of the last culture injected, either subcutaneously or intravenously, into a rabbit. From the lesions produced the organism was recovered after two days, and 2 c.c. of a 24-hour-old broth culture then injected into another animal. From each of the animals cultures were prepared and inoculated into ordinary broth and 2 per cent lactose broth, litmus milk, and slant agar, in order to find whether any cultural difference from the original appeared. Microscopical examinations were also made in each instance. The following strains were employed:

1. *Strept. lacticus* isolated from certified milk by the writer.

2. Another strain of the same organism. These two cultures were selected at random from a large number, and were typical of *Strept. lacticus* (*B. lactis acidi*, Leichmann).

3. *Strept. lacticus* obtained from Professor Harding, N. Y. Agricultural Experiment Station, Geneva, N. Y. This organism was to all appearances identical with the two previous ones in cultural and morphological characters.

[1] *Archiv f. Hyg.*, 1906, 56, p. 90. [2] *Ibid.*, p. 51.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 173.

[4] V. Lingelsheim in Kolle and Wassermann, *Handbuch der Pathogenen Mikroorganismen*, 1903, 3, p. 350

4. *Strept. lacticus* from a commercial starter.

5. *Strept. lacticus* also from a commercial starter. These two cultures, 4 and 5, agreed with typical *Strept. lacticus* in morphological and cultural characters.*

As controls and for comparison three strains of *Strept. pyogenes* were injected into rabbits in the same manner and in the same doses. Following are the sources of these cultures.

6. *Strept. pyogenes* isolated from the tonsil in a case of tonsilitis.

7. *Strept. pyogenes* isolated from a fatal case of peritonitis.

8. *Strept. pyogenes* isolated from a case of meningitis.†

These three strains possessed the usual cultural and morphological characters, but No. 8 coagulated milk very slowly.

After passage through animals the streptococci showed no noticeable cultural divergence from the original cultures. The only morphological difference was shown by *Strept. lacticus* No. 1, the original shape of this organism changing somewhat, and the individual cells in cultures from animals appearing rounder and smaller. Microscopical preparations from the lesions showed mostly diplococci and sometimes short chains. *Strept. lacticus* and *Strept. pyogenes* appeared alike.

A detailed description of the experiments follows:

1. *Strept. lacticus* (from milk): This strain was passed through 10 rabbits successively. A uniform amount each time (2 c.c.) was injected subcutaneously with the usual antiseptic precautions. The first rabbit developed an induration at the point of inoculation. The organism was recovered from the serous exudate after two days. The second rabbit was inoculated with a 24-hour-old broth culture of the recovered streptococcus in the same manner. It developed a small abscess. The pus was of a tough viscous consistency. The organism was recovered from this pus and a third animal inoculated with a culture therefrom. These methods of inoculation and recovery of the bacteria were pursued throughout all succeeding experiments. The pus pocket increased in size and rapidity of development with each succeeding rabbit. The fifth animal died in 29 days. Numerous pus centers were found under the skin, and purulent exudates were seen in the pleural and pericardial cavities. The ninth rabbit died in 16 days from a general streptococcus infection. There were fibrino-pur-

* Seven commercial starters were obtained from the manufacturers and examined bacteriologically. Five of these consisted of practically pure cultures of *Strept. lacticus*, while two were not pure cultures but contained streptococci in large numbers. After preparing pure cultures from all starters by plating, they were studied and found to possess all the characters of *Strept. lacticus*. The streptococci obtained from two of the starters were very feeble, growing but poorly and coagulating milk much more slowly than *Strept. lacticus* usually does.

† These strains were kindly given me by Dr. H. T. Ricketts and Mr. J. E. Tyree, Department of Pathology, University of Chicago. They were isolated less than two weeks before the commencement of my experiments

ulent exudates in the pleural, pericardial, and peritoneal cavities, and numerous purulent foci under the skin. Streptococci were recovered from the pleural and peritoneal cavities, heart's blood, kidney, spleen, and subcutaneous pus. The 10th rabbit died after nine days. The lesions were similar to those described in the foregoing. All these rabbits lost progressively in weight and presented an emaciated appearance. Five out of the 10 rabbits inoculated gradually recovered. Several, at the time of writing, still have a subcutaneous pus pocket and their emaciated appearance gives rise to the conjecture that they may finally die. In two instances no lesions developed at the point of inoculation. These rabbits died after a lapse of four weeks with the phenomena of general infections of the same nature as described, except that no noticeable change took place at the point of inoculation.

The same streptococcus (No.1) from the original culture was injected intravenously into a rabbit. The animal lost weight slowly and finally died after 25 days A large subcutaneous pus pocket had formed in the neighborhood of the hip on the right side, coincident with numerous pus foci of the same nature in other parts of the body. The pleural and peritoneal cavities were filled with a sero-fibrinous exudate and the pericardium showed a fibrino-purulent exudate. The organism was recovered from the pus, pleural, peritoneal, and pericardial cavities, and the heart's blood. From a broth culture prepared from the heart's blood a normal rabbit was injected intravenously and likewise a rabbit of the same weight, which had been previously inoculated subcutaneously with the same organism, but had recovered from the effects to all appearances. The former died in 17 days, the latter in six. The lesions were the same in both cases and identical with those described.

2. *Strept. lacticus* (No. 2, from milk): This strain produced no local effect. The animal after several days commenced to carry the head bent toward the left side, and when touched would walk around in a circle. When put on its back it regained its foothold only after several strenuous efforts. The animal seemed gradually to recover its normal condition, although it was considerably emaciated. Quite unexpectedly, after about six weeks, it died. Postmortem examination showed an enormously large amount of whitish semi-fluid pus in the pleural cavity. No other lesions could be detected. Streptococci were recovered from the pus and the heart's blood. Possibly the twisting of the head was due to an affection of the central nervous system by a toxin produced by the streptococcus. Similar observations have been recorded, among others by v. Lingelsheim.[1]

3. *Strept. lacticus* (from N. Y. Agricultural Experiment Station): The results of subcutaneous inoculation were quite similar to those described, excepting that the virulence increased somewhat more rapidly. The seventh rabbit died in 16 days, the eighth after 10 days.

4. *Strept. lacticus* (from a commercial starter): This organism seemed more virulent than any of the former three. The first rabbit developed a pus pocket at the point of inoculation of about an inch in diameter. The pus was of a thick, ropy consistency. An erysipelas-like reddening of the skin developed in the third rabbit at the point of inoculation. This reddening spread rapidly for four or five days, then came to a standstill, and finally disappeared. A large area of necrotic tissue formed in the fourth animal. This one also finally recovered. The seventh rabbit died on the ninth day after inoculation with the same postmortem picture as seen

[1] *Loc. cit.*

in the other animals. Here also streptococci were recovered from the pleural and pericardial cavities and the heart's blood.

5. *Strept. lacticus* (from a commercial starter): This strain was injected subcutaneously but produced no local effect. The rabbit suffered from a nervous trouble similar to that described under No. 2. The animal carried the head bent over to one side but gradually seemed to recover. It died after several weeks. No postmortem lesions whatever could be detected.

6, 7, 8. *Strept. pyogenes:* The three varieties of *Strept. pyogenes* produced the same effect on rabbits as *Strept. lacticus*, except that death occurred in a shorter time. *Strept. pyogenes* from tonsilitis (No. 6) caused the sixth rabbit to die in seven days. *Strept. pyogenes* from peritonitis caused the third rabbit to die in eight days, and *Strept. pyogenes* from meningitis produced the death of the fourth rabbit in seven days. The lesions were in every respect identical with those brought about by *Strept. lacticus*. By a fortunate coincidence the 10th rabbit inoculated with *Strept. lacticus* (No. 1) and the third rabbit inoculated with *Strept. pyogenes* from peritonitis (No. 7) died at the same time, the former after nine, the latter after eight days. Postmortem examinations were made simultaneously. The pathological conditions in the two animals were strikingly alike. Pus pockets under the skin in various places, the usual pericarditis, and the purulent exudate in the pleural cavity revealed identical conditions in both cases. Streptococci were recovered in both cases from the affected parts.

At the end of these experiments the different cultures from each of the animals were cultivated on slant lactose agar, plain and lactose broth, and litmus milk. It was noted that no change in the reactions on these media was effected by the passage through animals.

The conclusion seems justified that *Strept. lacticus*, by prolonged habitation in the body of the rabbit, is able to become virulent in large measure and approximate, if not equal, the virulence for this animal of typical *Strept. pyogenes* from human affections. It must be taken into consideration that the varieties of *Strept. pyogenes* employed in these experiments were recently isolated from pathological lesions. The streptococci from milk (Nos. 1, 2, and 3) had possibly lost in virulence by nine months' cultivation on artificial media. The organisms from commercial starters, although freshly isolated from the starters, probably had been in artificial cultivation for an indefinite length of time.

The negative results of inoculation experiments with milk streptococci by some observers may be explained in two ways. First: The lesions are often, especially in the beginning, insignificant and are easily overlooked. Second: Since lesions are insignificant with large doses it is quite possible that small doses are unable to create

any noticeable disturbances. Summarizing the results of the experiments described the following points seem established.

1. *Strept. lacticus* is closely related to *Strept. pyogenes*, not only morphologically and culturally, but also in pathogenic property.

2. By repeated passages of *Strept. lacticus* through rabbits its virulence is gradually increased. After five or more passages *Strept. lacticus* in subcutaneous injections becomes fatal in doses of 2 c.c. of 24-hour-old broth cultures. The same amount injected intravenously proved fatal to the first animal.

3. The lesions produced by *Strept. lacticus* in rabbits are of the same kind and extent as those produced by *Strept. pyogenes* from pathological conditions in human beings.

The writer acknowledges his obligation to Professor Edwin O. Jordan for advice and assistance in this work.

A STUDY OF THE PNEUMOCOCCUS, WITH ESPECIAL REFERENCE TO THE INULIN TEST.*

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THE work reported in this paper was carried on under the direction of Drs. William H. Park, and A. W. Williams, and consists of a study of some of the pneumococcus strains isolated at the Research Laboratory during the period between November, 1904, and August, 1905. The system of designating the strains is that used by Drs. Park and Williams in the report made to the Pneumonia Commission,[1] the description of the strains here mentioned being included in that report.

The organisms have been cultivated during the time since isolation upon blood agar, with occasional transfers to serum broth, or calcium broth. The possibility of contamination by similar organisms, during so long a period of cultivation on artificial media, has, of course, to be considered in judging results, but it is not thought that any contamination has taken place; first, because of the care exercised in transferring cultures and watching results; second, because where changes have occurred in the different strains they have been of a similar character, indicating a systematic tendency, and third, because in some cases, where a change has taken place, it has been possible to observe the different stages leading toward it, in a graduated series.

Very few strains are now found to be typical, morphologically. The majority show very small organisms and increased chain formation, namely, a more or less marked approach toward a streptococcus type. With some strains this change seems to be a permanent one, the organisms having a definite streptococcus morphology in all media; with others the change is but temporary, to be followed in later cultures by a return to a characteristic pneumococcus type, and with a few strains, while an occasional atypical culture is seen,

* Received for publication September 3, 1906.

[1] *Jour. Exper. Med.* 1905, 7, p. 403.

the majority of cultures now show a morphology as typical as when the organisms were first isolated more than a year ago.

Table 1 gives the results of a study of morphology and inulin coagulation made during January, 1906. Sixty-one strains were studied at this time, selected at random from the laboratory stock. Since but one inulin test was made with each strain in this series of experiments, only general conclusions can be drawn as to inulin coagulation from this part of the work, in view of the great irregularities later observed.

TABLE 1.

SUMMARY OF INULIN TESTS MADE JANUARY, 1906, UPON STRAINS OF PNEUMOCOCCI ISOLATED AT DIFFERENT PERIODS BETWEEN NOVEMBER, 1904, AND MARCH, 1905, COMPARED WITH SIMILAR TESTS MADE SOON AFTER ISOLATION. ALL POSITIVE IN ORIGINAL TESTS.

	Coagulated not later than in original test		Coagulated later than in original test		Not coagulated this time though coagulated in original test		Total
	Char.	Not Char.	Characteristic	Not Char.	Char.	Not Char.	
Pneumonia Cases	8_{1001} *9_{1001} 13_{1001} 46_{1001} 56_{1112} 60_{1001} 82_{1322}	18_{1122} 77_{1001}	15_{1112} 3d later 39_{1001} 2d " *47_{1122} 2d " 57_{1122} 8d " 83_{1001} 10d "	22_{1001} 2d later	4_{1001} 62_{1112} 67_{1001} 72_{1001} 75_{1001} 76_{1001} 98_{1122}	20_{1122} 21_{1122} 36_{1001} *47_{1001} 73_{1001}	
	7	2	5	1	7	5	
	9		6		12		27
Normal Cases	$N41_{1122}$ $N124_{1001}$ $N135_{1122}$ $N136_{1212}$	$N106_{1112}$ $N111_{1001}$ $N127_{1112}$ $N113_{1001}$	$N89_{1122}$ 8d later $N99_{1122}$ 9d later		$N23_{1122}$ $N51_{1122}$ $N52_{1122}$ $N59_{1122}$ $N91_{1222}$ $N100_{1122}$ $N109_{1112}$ $N130_{1112}$	*$N10_{1112}$ $N11_{1122}$ $N43_{1122}$ $N45_{1001}$ $N107_{1111}$	
	4	4	2	0	8	5	
	8		2		13		23
Miscellaneous Cases	C & $D4_{1001}$ $Q5_{1122}$	$C23_{1112}$ $Me1_{1122}$	$C5_{1122}$ 1d later	$Me2_{1112}$ 3d later	C&$D8_{1001}$ $T3_{1122}$ $Q3_{1001}$ $C23_{1122}$	*$C4_{1122}$	
	2	2	1	1	4	1	
	4		2		5		11
Total							61

**Pneumococcus Mucosus.*

TABLE 2.

Synopsis of Characteristics of Pneumococcus Cultures before Passage through Animals, and after Passage through Last Series.

Original Culture			Original at time of Isolation	Original January, 1906	Original before Inoculation May, 1906	Mice					Original June, 1906
						1	2	3	4	5	
*4_{1001}	Morphology	Typ.	+	+∓	∓	+∓	Discarded	+	Lived		+
		Atyp.									
		Caps.	+			−		+			
		Inulin	+	−	−	−		+			−
†16_{1112}	Morphology	Typ.	∓	∓+	+				Discarded	+	∓
		Atyp.				+	+	∓+			
		Caps.	?			?	−	−		Indic.	
		Inulin	+	−	−	∓	∓	∓		+	+
36_{1001} (No. 1)	Morphology	Typ.	+			Discarded					
		Atyp.		+	+		+	+	+	Lived	+
		Caps.	+				−	−	No org		
		Inulin	+	−	−		−	−	−		−
36_{1001} (No. 2)	Morphology	Typ.	+	+	+	Not inoc.					+
		Atyp.									
		Caps.	+								
		Inulin	+	+	+						+
47_{1001}	Morphology	Typ.	+	+	∓+	∓+	+	∓+	+	+	+
		Atyp.									
		Caps.	+			−	−	−	−	No org	
		Inulin	+	−	−	∓	−	∓	∓	−	+
76_{1001}	Morphology	Typ.	+	∓							
		Atyp.			+	+	+	+	+	+	+
		Caps.	Indic.			Indic.	−	−	−	−	
		Inulin	+	−	−	+	∓	−	∓	∓	−
$N23_{1122}$	Morphology	Typ.	+	+∓	+	+	+	∓+	∓	+	+
		Atyp.									
		Caps.	+			?	+	−	∓+	+	
		Inulin	+	−	−	+	+	∓		+	+
$N91_{1222}$	Morphology	Typ.	+	∓	+	+	+	+	Lived		+
		Atyp.									
		Caps.	+			+	+	+			+
		Inulin	+	−	−	+	+	+			+

*+∓=Plate typical, serum broth not characteristic.
†∓+=Plate not characteristic, serum broth typical.
1001=Organism isolated from plate.
1122= " " " 1st rabbit.
1222= " " " 2d "
1112= " " " mouse.

Twenty-seven strains were studied which came originally from cases of pneumonia. Nine of these coagulated inulin not later than in original tests. Seven were fairly characteristic morphologically, two not characteristic; six coagulated later than in original tests, five characteristic, one not characteristic; while 12 did not coagulate in these cultures, though all gave a positive reaction when first tested.

When first studied at the time of isolation, 21 of the above were typical, six fairly typical.

Twenty-three strains from normal cases were studied, eight of these coagulating inulin not later than in first tests, four being characteristic, four not characteristic; two coagulated later than in first tests, both characteristic, and 13 did not coagulate at this time, although positive in original tests. When first studied 13 were typical, four not characteristic, and six atypical.

TABLE 3.

TOTAL INULIN TESTS: 4 CASES.

CASE	STRAIN		DATE OF CULTURE					TOTAL RESULTS			TOTAL TESTS
			At Time of Isolation	January, 1906	March, 1906	May, 1906	June, 1906	−	∓	+	
N23 1122	Original strain	−		1	2	2	3	8			11
		∓					1		1		
		+	1				1			2	
	M. A.	−			2			2			2
	R. A.	−			1	1		2			4
		∓			1				1		
		+			1					1	
	R. B.	−				1	3	4			9
		∓					1		1		
		+			1		3			4	
	M1	−					3		3		6
		+					3			3	
	M2	−					4		4		7
		+					3			3	
	M3	−					1	1			3
		∓					2		2		
		+									
	M4	−					1	1			3
		∓					2		2		
		+									
	M5	−					1	1			5
		∓					2		2		
		+					2			2	
	M. R. 1	−					4	4			9
		∓					3		3		
		+					2			2	
	M. R. 2	−					3		3		4
		∓					1			1	
		+									
	Total							23	22	18	63

NOTE.

Total coagulating cultures in black-face figures.
M. A.=first mouse inoculated.
M. 1.= " " " of second series.
M. M. 1.=first mouse inoculated from mouse of first series.
M. R. 1.= " " " from rabbit.
R. A.=first rabbit inoculated.
R. R.=rabbit inoculated from rabbit.

TABLE 3.—*Continued.*

Case	Strain		Date of Culture: At Time of Isolation	January, 1906	March, 1906	May, 1906	June, 1906	Total Results: −	∓	+	Total Tests
N91₁₂₂₂	Original strain	−		1	2	3	6	12			15
		∓									
		+	3							3	
	M. A.	+			1					1	1
	M. B.	+			2					2	2
	M. C.	−				1		1			8
		∓			1		1		2		
		+			1	1	3			5	
	R. A.	+			2					2	
	R. B.	−					1		1		5
		∓									
		+			1	1	2			4	
	M1	−					2	2			2
		∓									
		+									
	M2	−					3	3			3
		∓									
		+									
	M3	−					2	2			8
		∓					2		2		
		+					4			4	
	M. M. 1	−									8
		∓									
		+					8			8	
	M. M. 2	−					1	1			7
		∓					2		2		
		+					4			4	
	M. R. 1	−									4
		∓									
		+					4			4	
	M. R. 2	−									5
		∓					1		1		
		+					4			4	
	Total							21	8	**41**	70

TABLE 3.—*Continued.*

Case	Strain		Date of Culture: At Time of Isolation	January, 1906	March, 1906	May, 1906	June, 1906	Total Results: −	∓	+	Total Tests
4₁₀₀₁	Original strain	−		1	2	3	6	12			13
		∓									
		+	1							1	
	M. A.	−			2			2			2
	M1	−				2	2	4			4
		∓									
		+									
	M3	−				1	1		2		4
		∓				1	1			2	
		+									
	Total							18	2	**3**	23

TABLE 3.—*Continued.*

CASE	STRAIN	DATE OF CULTURE	MORPHOLOGY			INULIN					TOTAL TESTS
			Typical	Atypical	Capsule	Total Results					
						−	Sl.	∓	+	+†	
N62₁₁₂₂	Original	Mar. '05	∓	+	−	4					4
	"	Jan. '06	∓			1					1
	"	Mar. '06		+		1					1
	*R. A.	" '06	∓		No org.				1		1
	R. B.	" '06		+	No org.	1					1
	M. A.	" '06		+	+	1					1
	Original	May, '06		+		1					1
	"	June, '06		+		3	1	2	2		8
	R. A.	" '06		+		2		2	1		5
	†M. R. 1	" '06	±		—	1	1	10	2		14
	M. R. 2	" '06		+	No org.	2		3	1		6
	M. R. 3	" '06	±		−	1		2	1		4
	M. R. 4	" '06		+	Indic.	1		3	1		5
	R. R.	" '06		+	−				3	1	4
	Total					19	2	22	12	1	56

*Plate atypical; serum broth typical.
† " typical; " " atypical.

Of the 11 strains from miscellaneous cases, four coagulated inulin, not later than when first studied, two characteristic, one not characteristic; and five failed to coagulate, although positive in original tests, four of these characteristic, one not characteristic. When first studied six were typical, five not characteristic.

Unless extremely small, size of organisms has not been considered in this division into characteristic and non-characteristic, the classification being based upon the general morphological picture.

In order to ascertain the effect upon the inulin fermenting property produced by passage of the organism through animals, a certain number of strains were selected for further study, and in March, and again in June, of the present year, these were inoculated into mice and rabbits.

In these experiments no attempt was made to ascertain the smallest lethal dose, but all strains were found to have lost virulence, very large doses being required to kill the animals. In the last series of mouse inoculations (Mouse 1, Mouse 2, etc.) the organism was passed directly from one animal to another; the heart being divided, the chest cavity then washed with sterile water, and this inoculated intraperitoneally, cultures being made at the same time.

By this method, a high degree of virulence was developed in some cases, mice dying within six to eight hours after inoculation, with profuse cultures of the pneumococcus given by the heart's blood.

Table 2 gives a synopsis of morphology and inulin coagulation with the original strains and after passage through a second series of animals.

Table 3 gives the complete record of inulin tests made with four strains, the results in these cases being typical of those given by the other cases studied.

Of the comparatively small number of cultures studied only two have coagulated the inulin medium in recent tests which failed to coagulate it when first isolated. These two, 16_{1112} and N 62_{1122}, were not included in the January table.

No. 4_{1001} originally a large, typical pneumococcus, showing capsules and coagulating inulin, has in these tests shown a variable morphology, with loss of capsules and inulin-fermenting power, but after passage through Mouse 3 there is again a typical morphology, with capsules, and two cultures showing complete and two partial inulin coagulation. Of a total of 23 inulin tests, three are positive, two partial, and 18 negative. To this list may be added at least six coagulations not recorded, made during the first few months after isolation, when the organism was frequently tested and always gave prompt positive results. The negative tests have all been made since the beginning of January.

No. 16_{1112} which has shown a mixed streptococcus and pneumococcus morphology throughout cultivation, and was negative to inulin when first tested, now gives positive inulin coagulation in cultures, both from the original strain and after passage through Mouse 5. Of the total inulin tests three are positive, eight partial, and 20 negative.

No. 36_{1001} has been of special interest. It was originally a very typical pneumococcus, showing capsules, coagulating inulin promptly, and very virulent for animals. It now shows in two series of cultures from the same strain, entirely opposite characteristics. The one series consists of later transfers from a series which in the summer of 1905 was carried on for many generations on special media namely: horse blood agar, rat blood agar, rabbit blood agar, and mouse blood agar; cultures being then tested for virulence and inulin coagulation. Originally virulent for mice in doses of 1/1,000,000 c.c., rabbits 1/5,000, and rats 1/10 c.c., all cultures were now found to have decidedly lost in pathogenic power for these animals. Inulin was still promptly coagulated in seven cultures. Tried again in October of the same year, the cultures were found to be non-virulent for rabbits, in 4 c.c. doses and no longer killed mice unless given in large doses of a strong emulsion. No further tests of virulence have been made with this series. The cultures are still fairly typical morphologically, have recently coagulated inulin, and have given 12 positive coagulations during the past six months.

The contrasting series of 36_{1001} consists of the regular laboratory stock cultures. In these a complete change has taken place, and the organism now grows as a typical streptococcus in all media, is cultivated with difficulty, and shows no reaction with inulin serum water. This change has been a gradual one, all degrees of morphology

having been observed between the two types of organisms, and repeated fishing from atypical colonies have given cultures of a similar mixed growth. Of 56 total inulin tests, 23 have been positive, 33 negative.

No. 47_{1001} is of peculiar interest on account of its morphological changes. Originally a typical *Pneumococcus mucosus*, it has shown in subsequent cultures a varying morphology, sometimes of a typical pneumococcus and again of a streptococcus type, with occasional reversions to the original *Pneumococcus mucosus*, the latter form generally appearing only after passage through animals, especially mice, or when a fresh transfer is made after a long resting period. After persisting for a varying number of transfers (from one or two to eight) the *Pneumococcus mucosus* form is again lost, the organism reverting to the pneumococcus, the streptococcus, or to mixed types. Table 4 shows the variations observed after continued cultivation on various media, according to plan described under 36_{1001}. In the transfers following those given in the table all the horse blood agar cultures showed the *Pneumococcus mucosus* form, which persisted for eight weekly transfers, the pneumococcus, the streptococcus, or the mixed forms then reappearing. In January last a typical *Pneumococcus mucosus* was again obtained from this series, the form persisting for several transfers.

The changes of form observed in this organism are in unison with the results previously obtained at this laboratory, and are a confirmation of the intimate connection and interchangeable nature of the pneumococcus and *Pneumococcus mucosus* types of organism.

No. 47_{1001} was originally virulent for mice in 1/100,000 c.c., rats, 1/100 c.c. Tested after growth on the various blood agars, a loss of virulence was found in all cultures. Some difference was noticed in cultures according to the media upon which growth had been carried on, but upon the whole the results were irregular, and without further tests no definite conclusion could be drawn upon this point.

The coagulation of inulin, while at first prompt, has since been irregular with this organism. Of 54 inulin tests, 13 are positive, six partial, and 35 negative.

No. 76_{1001} is another organism which has shown a marked change. Originally a typical pneumococcus, coagulating inulin promptly, in these tests it has appeared as a characteristic short streptococcus, and with the exception of two positive coagulations from Mouse 1, and four partial reactions from other animals, all recent inulin tests have been negative. Of 31 total inulin tests three are positive, four partial, and 24 negative.

N 62_{1122} has shown features of peculiar interest and in the summary results have been given more in detail. With the exception of a few cultures which more nearly approach the pneumococcus type, this organism has presented the appearance of a typical streptococcus during the entire period of cultivation. It was negative to inulin when first isolated and in many subsequent tests, but in the recent series of experiments coagulations have been obtained with cultures from each animal, and also from the original strain. Of 56 inulin tests, 13 have been positive, 24 partial, and 19 negative.

N 23_{1122} and N 91_{1222} were both originally typical pneumococci, showing capsules, coagulating inulin promptly, and virulent for animals. With the exception of an occasional culture showing increased chain formation, both strains have been typical in morphology throughout, N 91_{1222} especially showing the most typical large pneumococci, but the record of inulin tests shows that with both strains many cultures

have failed to coagulate inulin. After passage through animals coagulation again occurs, and recently positive inulin tests have also been obtained from the original strain of N 23. N 23_{1122} in 63 inulin tests shows 18 positive, 22 partial, and 23 negative; N 91_{1222} in a total of 70 tests gives 41 positive, eight partial, and 21 negative; we have therefore but 35 per cent and 58.5 per cent respectively of positive inulin tests for these two very typical strains of pneumococci.

TABLE 4.

Showing Variations in Morphology (Pneumococcus and Pneumococcus Mucosus Forms), and Inulin Coagulation of 47_{1001} in August, 1906, after Continual Cultivation on Special Media. First Series Transferred Daily; Second Series after Interval of Nine Days.

Series	Date of Culture	Medium	Total Transfer No.	Transfer on Special Medium	Typ. Pneumococcus Mucosus		Typ. Pneumococcus		Inulin Coagulation
					Growth	Smear	Growth	Smear	
Horse Blood Agar — Transferred daily	8/9	Horse bl. agar	90	30	∓	∓			
	8/9	Serum broth	90			+			
	8/10	Inulin				+			∓
	8/11	Bl. agar plate					∓	∓	
Horse Blood Agar — Transferred 9th day	8/9	Horse bl. agar	85	25	+	+			
	8/9	Serum broth	85					+	
	8/10	Inulin				+			+
	8/11	Bl. agar plate			+	+			
Rabbit Blood Agar — Transferred daily	8/9	Rabbit bl. agar	101	41	∓	∓			
	8/9	Serum broth	101			∓			
	8/10	Inulin				∓			−
	8/11	Bl. agar plate					+	+	
Rabbit Blood Agar — Transferred 9th day	8/9	Rabbit bl. agar	96	35	∓	∓			
	8/9	Serum broth	96			∓			
	8/10	Inulin				∓			+
	8/11	Bl. agar plate					∓	∓	
Rat Blood Agar — Transferred daily	8/9	Rat bl. agar	101	40	∓	∓			
	8/9	Serum broth	101			∓			
	8/10	Inulin				∓			+
	8/11	Bl. agar plate					+	+	
Rat Blood Agar — Transferred 9th day	8/9	Rat bl. agar	96	35	+	+			
	8/9	Serum broth	96			∓			
	8/10	Inulin				∓			+
	8/11	Bl. agar plate					∓	∓	
Mouse Blood Agar — Transferred daily	8/9	Mouse bl. agar	102	11	∓	∓			
	8/9	Serum broth	102			∓			
	8/10	Inulin				∓			+
	8/11	Bl. agar plate			∓	∓			
Mouse Blood Agar — Transferred 9th day	8/9	Mouse bl. agar	97	6	∓	∓			
	8/9	Serum broth	97			∓			
	8/10	Inulin				∓			+
	8/11	Bl. agar plate					∓	∓	

Blood agar and serum broth cultures made from blood agar of August 8, in each case. Plates made from inulin cultures.

Considered as a whole, it will be seen that the entire series of inulin tests here reported is characterized by a marked irregularity as to coagulation results, this being the case not only with atypical, but also with typical strains.

As the tables show, passage through animals seems generally to have a favorable influence upon the inulin fermenting power of the pneumococcus, an effect best shown in the strain from N 91.

The number of organisms inoculated into the inulin medium is often found to be an important factor, as coagulations have several times been obtained by the use of a strong emulsion of organisms after many negative results with cultures of ordinarily abundant growth. This, however, is not an invariable rule, as good coagulations have been obtained in some cases from cultures showing a very poor growth, while other cultures of the same inulin lot, containing a heavy growth of the same organism, have failed to coagulate.

The growth of the organisms in inulin has been studied in all cases, either in smears or by blood-agar streak plates, whenever possible, by both methods, and no cultures have been included in the report which have not been found to contain an abundant number of organisms; the large number discarded on account of insufficient growth causing the irregularity in the number of tests recorded for each strain. All inulin cultures were incubated for two weeks before being classed as negative.

The inulin serum water used in these tests was made according to the usual method, with one-third ox serum, two-thirds distilled water, and 1 per cent inulin powder, each lot being tested with laboratory stock cultures before being used in these experiments. The inulin powder used in some of the earlier work was extracted by Dr. Gibson at the Research Laboratory from dandelion roots, a preparation which gave very good results. For all the other tests the medium used was prepared from Merck's inulin (white). Various stocks of this powder, procured at different periods were found to differ greatly, and one whole stock had to be discarded, as no reliable tests could be obtained from it. In all of the work done in March and subsequently, but one stock of Merck's white inulin has been employed, 5 c.c. of the inulin serum water being inoculated with $\frac{1}{2}$ c.c. of culture in each test. The greatest variation has been found between lots of inulin serum water made at different dates from this one inulin powder, and from the same or different lots of ox serum. Tubes inoculated with the same strain show all stages between entirely negative and positive results and an equal irregularity is found in many cases when tubes of the same or different lots of inulin medium are inoculated simultaneously with the contents of the same culture tube. Some difference was noticed between lots of inulin medium according to the supply of ox serum used, but at the end of the time-limit differences based upon this distinction were either very slight or no longer noticeable. Table 5 gives the results of the tests made with N 23_{1122} and N 91_{1222} in five lots of inulin medium made of the same inulin powder, and two different lots of ox serum.

TABLE 5.

Results of Tests with Five Lots of Inulin Serum Water. All Made from Same Inulin Powder (Merck's White) from Two Lots of Ox Serum—2 Cases.

Case	Strain	Tests Grouped under Lot of Inulin Medium Used											Total Tests
		Tubes Inoculated						Results					
		1st Ox Ser. Lot			2d Ox Ser. Lot			Coagulation					
		1	2	3	4	5		−	SL	+	+	+ +	
N23 1142	Orig.	1	1	1	1	1		1 1 1		1	1		5
	M 1	1		1	3	1				2 1	1 1	1	6
	M 2	1		2	3	1				3 1	1	1 1	7
	M 3		1		1	1		1		1 1			3
	M 4		1		1	1		1		1 1			3
	M 5		1	1	2	1		1		1 1	1	1	5
	R. B.			1	1	3		2		1	1 1		5
	MR 2		1	3	3	3		1 2 2		2 1	1 1		10
	MR 2			1 •	1	1					1 1 1		3
	Total.....	..	..	..	..	..	47	13		18	**12**	**4**	47
Tests Grouped According to Ox Serum used	1st Ox Serum	3	5	10				1 5 1		2	5	2 2	
							18	7		2	**5**	**4**	18
	2d Ox Serum				16	13		4		11 9	5 2		
							29	4		18	**7**		29

TABLE 5.—*Continued.*

CASE	STRAIN	TESTS GROUPED UNDER LOT OF INULIN MEDIUM USED											TOTAL TESTS
		Tubes Inoculated						Results					
		1st Ox Ser. Lot			2d Ox Ser. Lot			Coagulation					
		1	2	3	4	5		−	SL	+	+	+⁺	
N 91222	Orig.	1	1	1	1	2		1 1 1 1 2					6
	M 1				1	1		1 1					2
	M 2		1		1	1		1 1 1					3
	M 3		1	3	3	1		1 1	1		1 3 1		5
	R. B.			1	1	1				1	1 1		3
	MR 1		1	2	1						1 1 1	1	4
	MR 2			3	2	2				1	2 1 2	1	7
	MR 3			1								1	1
	M. D.		1	2		1				1	2 1		4
	MM 1			4	3	1					3 3 1	1	8
	MM 2		1	2	1	2		1		1 1	1 1	1	6
	Total.....	..	..	..	..	..	52	14	1	5	27	5	52
Tests Grouped According to Ox Serum Used	2d Ox Serum	1	6	19				1 4 2	1	1 1	1 10	5	
							26	7	1	2	11	5	26
	1st Ox Serum				14	12		3 4		2 1	9 7		
							26	7		3	16		26

A few cultures of streptococci recently obtained from various pyogenic sources were inoculated into animals, and the morphology and inulin growth in media studied as above. One of these strains resembles a pneumococcus, and one a *Pneumococcus mucosus* in several cultures, and all show capsules after passage through animals. Table 6 gives a summary of results and shows that all inulin cultures remain negative. These experiments are too few to be of any importance, but are given as controls, and because it seems possible that further study in this direction may establish the pneumococcus nature of some organisms now classed as streptococci.

TABLE 6.

Synopsis of Characteristics of Streptococcus Cultures before and after Passage through Animals.

Case			Original Strain	Rabbits	Mice					
					M. A.	M. B.	M. C.	M. D.	M. E.	M. F.
* Trachoma	Morphology	Typ.	+	+	+	+	∓	∓	∓	∓
		Atyp.		+	−	+	+	+	+	+
		Caps.								
		Inulin	−	−	−		−	−	−	−
† Empyema	Morphology	Typ.	+	∓	+	∓	+	Lived		
		Atyp.		+	+	+	+			
		Caps.								
		Inulin	−	−	−	−	−			
Puerperal Septicemia	Morphology	Typ.	+	+	+	Not inoculated				
		Atyp.		+	?					
		Caps.								
		Inulin	−	−	−					

* Many cultures suggest resemblance to *Pneumococcus mucosus*, most marked in cultures from Mouse A.

† Cultures from rabbit and from Mouse B suggest resemblance to pneumococcus.

Tests of the various lots of inulin powder and of the inulin medium before inoculation have so far led to no explanation of the varying coagulation results. No differences could be detected between the several stocks of inulin powder, the rejected stock appearing to be identical with the rest except that coagulations could not be obtained with the medium made from it. Nine lots of the inulin medium made at different dates from good powder were tested for acidity before inoculation with organisms. Five c.c. titrated with N/50 NaOH and phenolphthalein were found to vary between neutral and 1.4 acid, the majority being about 0.2 acid.

Flasks containing 50 c.c. of inulin medium after titration for initial acidity were inoculated each with 5 c.c. of one of the cultures of the strains studied, and inoculated for 10 days. Some of the contents of these flasks was pipetted out every one or two days and tests made of reactions and growth in the inulin medium. Two flasks inoculated with N 91_{1222} showed coagulation nearly complete in 24 hours, with acidity 2.2 and 2.8 respectively, organisms very characteristic, and no further tests possible because of coagulation. All flasks inoculated with other organisms gave a negative coagulation result, and showed practically no increase in acidity, while at the same time the count of plate colonies showed a good growth of organisms present. Table 7 gives the results of these tests with four of the previously mentioned strains and also with one strain of streptococci; the highest acid production being given in each case compared with the reaction before inoculation, and with the highest plate count.

TABLE 7.

RESULTS OF TITRATION AND PLATE COUNT WITH NON-COAGULATION OF INULIN FLASK CULTURES—5 C.C. TITRATED WITH N/50 NaOH.

STRAIN	TITRATION		PLATES
	Before Inoculation	Highest Acidity	Highest Count to 1 c.c.
16_{1112}	1.4	1.9	98,102,440
47_{1001}	1.1	1.7	128,142,000
$N23_{1122}$	0.4	0.9	45,896,050
$N62_{1122}$	0.2	1.7	73,710,000
Streptococci	0.2	1.8	33,015,000

Further work in this direction was contemplated but has not yet been carried out, the same being true of plans to attempt to ascertain the nature of the acid produced by the growth of the pneumococcus in the inulin medium.

CONCLUSIONS.

Many strains of pneumococci, after longer or shorter periods of cultivation on artificial media, are found to undergo decided changes in morphology, virulence, and power to ferment inulin.

These changes may be temporary, disappearing when the organisms are placed under favorable conditions, but in some cases they seem to be permanent, the organisms having apparently undergone a complete change from their original characteristics.

The change in morphology is toward a more or less complete approach to a streptococcus type, some tendency in this direction appearing in certain cultures of every organism studied.

The presence of the pneumococcus and of the *Pneumococcus mucosus* types in organisms of the same strain shows the close relationship and interchangeable character of these two types.

The change in virulence corresponds with that usually found after long artificial cultivation. By transfers directly from animal to animal a rapid increase of virulence is developed.

The change in inulin fermenting power represents a marked irregularity of reaction constituting the chief feature in this series of tests which, including those made at time of isolation, consists of 452 inulin tests made with cultures from 63 strains of organisms.

A large number of negative tests have been given by very typical pneumococci and, on the other hand, many coagulations have been obtained from cultures of a definite streptococcus type.

Great variations have been found between different stocks of inulin powder and also between different lots of inulin medium made from the same powder and inoculated with organisms of the same strain.

Passage through animals seems, in many cases, to have a favorable effect upon the inulin fermenting power of the pneumococcus.

The use of strong emulsions of organisms sometimes produces coagulation, where ordinarily abundant cultures give only negative results.

The conclusion drawn from these experiments is that while coagulation of inulin is thought to be valuable corroborative evidence in favor of the pneumococcus nature of an organism, yet the irregular nature of the reaction may make it a fruitful source of differences and errors in diagnosis if too much reliance is placed upon this test, since it is evident that no organism can be rejected as a pneumococcus because of one, or even of several, non-coagulating inulin cultures. Especially is this true of cultures which have been grown for some time on artificial culture media.

A STUDY OF LATENT AND RECURRENT MALARIAL INFECTION AND THE SIGNIFICANCE OF INTRA-CORPUSCULAR CONJUGATION IN THE MALARIAL PLASMODIA.*

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INTRODUCTION.

IN previous contributions [1, 2, 3, 4] I have considered the subjects of latent malarial infection and of the conjugation of the malarial plasmodia. In the present communication† it is my purpose to give in detail the data which I have collected to date regarding latent infection and recurrent infection, and to consider especially the significance of intra-corpuscular conjugation as it occurs in the malarial plasmodia.

The material upon which the observations and conclusions detailed in this repor are based consisted of 1,653 cases of malarial infection, 1,267 of which were observed at the U. S. Army General Hospital, Presidio of San Francisco, in American soldiers returning from the Philippine Islands, and 386 cases studied at Camp Stotsenburg, Pampanga Province, Philippine Islands. Of the latter cases 248 occurred in Americans, and 138 in Filipinos. Besides the cases mentioned a considerable portion of the data regarding latency and recurrence has been obtained from the observation of malarial infections contracted by American soldiers while in Cuba, and studied at the Simpson U. S. Army General Hospital, Fortress Monroe, Virginia, and at Camp Columbia, near Havana, Cuba.

A word as to the classification of malarial infections: Almost all authorities now admit the existence of three species of malarial plasmodia, the tertian, quartan, and estivo-autumnal. By many the estivo-autumnal plasmodia are divided into two varieties, the tertian and quotidian estivo-autumnal plasmodia, while by some the latter organism is subdivided into a pigmented and unpigmented quotidian. Other observers maintain that there is but one estivo-autumnal parasite, the variations observed in the temperature curve being due to variations in the time of the developmental cycle, which may be 24 or 48 hours in length. Personally I have adopted the classification of Marchiafava and Bignami[5], who divide the estivo-autumnal plasmodia into two varieties, the tertian and quotidian.

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The careful study of many hundred cases of estivo-autumnal malaria has conclusively proven to my mind the existence of a tertian and quotidian estivo-autumnal plasmodium. They can be differentiated morphologically, and the infections produced by them are, when uncomplicated, clinically distinct. Anyone who can differentiate the tertian and quartan plasmodia morphologically should have no trouble in distinguishing between the tertian and quotidian estivo-autumnal plasmodia if blood from the spleen, obtained by puncture, be examined. To one who has had the opportunity of studying a large number of cases of estivo-autumnal infection the two organisms can easily be distinguished by the forms occurring in the peripheral blood.

As regards the argument that the tertian and quotidian temperatures observed in estivo-autumnal malaria are due to the sporulation of a parasite which at one time completes its developmental cycle in the blood in 24 hours and at another time in 48 hours, I can only say that it is illogical and can as well be used in the case of the tertian and quartan plasmodia by those who still maintain that there is but one species of malarial plasmodium.

I have not been able to demonstrate the occurrence of a pigmented and unpigmented quotidian estivo-autumnal parasite. While it is not uncommon to observe only unpigmented quotidian plasmodia in the peripheral blood in estivo-autumnal infections, blood obtained by splenic puncture in such cases has always shown pigmented organisms, and I have yet to observe a case in which both the peripheral and splenic blood showed only unpigmented malarial plasmodia.

1. LATENT MALARIAL INFECTIONS.

By a latent malarial infection I understand one in which the plasmodia of malaria may be demonstrated to be present in the blood of an individual, but in which no clinical symptoms of the disease of sufficient gravity to attract attention are to be observed. The term should not be confined to those instances in which no symptoms of malaria have ever been present, for if the parasites be present in the blood in recurrent cases, between the attacks the disease is as truly latent as it may be before the initial one. In many latent infections some other disease may be present, and this is almost invariably true of latent malarial infections discovered in hospital practice. It is obvious that all cases of latent malarial infection are a great source of danger to others in localities where *Anopheles* mosquitoes are present.

Of the 1,653 cases of malarial infection upon which the statistics of this contribution are based, 424, or a little over 25 per cent, were latent infections. Of these 307 were in American soldiers or civilians, while 115 occurred in natives of the Philippine Islands.

As regards the species of plasmodium present, the 422 cases were divided as follows:

Tertian	110
Quartan	8
Estivo-autumnal—	
Tertian estivo-autumnal	272
Quotidian estivo-autumnal	25
Combined tertian and tertian estivo-autumnal	7
Combined tertian and quotidian estivo-autumnal	2
Total	424

In order to understand the significance of the above table it will be necessary to consider the latent infections of Americans and of natives of the Philippines separately.

Latent infections in Americans.—The latent infections in Americans, as regards the type of plasmodium present, were divided as follows:

Tertian	81
Quartan	0
Estivo-autumnal—	
Tertian estivo-autumnal	199
Quotidian estivo-autumnal	21
Combined tertian and tertian estivo-autumnal	4
Combined tertian and quotidian estivo-autumnal	2
Total	307

The 307 latent cases observed in Americans occurred, with but few exceptions, in soldiers invalided home from the Philippines. As is evident, the estivo-autumnal plasmodia are much more frequently encountered in these cases than any other species, 220 of the 307 latent infections in Americans being due to either the tertian or quotidian estivo-autumnal parasite. The occurrence of so large a proportion of estivo-autumnal infection is not due to any peculiarity in the type of plasmodia other than that the estivo-autumnal organisms are much more resistant to quinine than either the benign tertian or quartan, and for that reason more latent infections with these types of plasmodia are found. It is also undoubtedly true that in the Philippines, as in other tropical countries, the estivo-autumnal malarial fevers are more prevalent than tertian or quartan fevers.

The fact that estivo-autumnal infections are particularly apt to exist in a latent form is of great importance, as the diagnosis of such an infection may prevent a sudden pernicious attack; and it is also

important from an epidemiological standpoint, as an individual harboring the plasmodia is, or may become, a source of infection to the community in which he is living. It will thus be seen that the blood of every individual returning from a malarial region should be examined, both as a safeguard to himself and to the community in which he resides.

Frequency of latent infection in Americans.—Of the 1,297 cases of malaria observed in Americans 307 or nearly 24 per cent were latent infections. It should be remembered that in all of these cases there were absolutely no clinical symptoms of malaria present, and had it not been for the examination of the blood these men might have been sources of infection for weeks or months.

As showing the frequency of latent malarial infections and the importance of their recognition, I quote the following from a previous report upon this subject:

"In August, 1902, Company H, Sixteenth Infantry, U. S. Army, returned to San Francisco from the Philippines, having served in the Cagayan Valley, a notoriously malarial region in those islands. On August 16, 1902, this company, out of a total strength of some 60 men, had 14 men in hospital suffering from malarial infection, all having had chills since arrival in the United States. On account of this large proportion of infected men I believed that it would be advisable to make a blood examination of the entire company, and accordingly, on August 17, I examined the blood of every man in Company H, with the following results. Of the 47 men who were doing duty, including officers, I found that 27 presented some form of malarial parasite in their blood. Of these 27 cases 25 were infected with estivo-autumnal plasmodia; 13 showing crescents of the tertian estivo-autumnal type; 2 showing crescents and ring-forms of the quotidian estivo-autuminal type; and 10 showing crescents and ring-forms of the tertian estivo-autumnal parasite. Two of the cases showed nearly full-grown forms of the benign tertian plasmodium. Thus of a total strength of 60 men, 41, which includes those in hospital, showed some form of malarial infection, while 27, without presenting any symptoms of malarial infection, showed parasites in the blood."

A study of the blood findings in this company is of great significance, not only as showing the importance of a blood examination in persons returning from the Tropics, but because, as will be noticed, the species of plasmodium present most frequently is the most dangerous of the plasmodia, and because, in most instances, the crescent form is the one present. This form, as is well known, is intended to complete its development in the mosquito and thus these men would be able to infect mosquitoes of the genus *Anopheles* wherever they might go. In this way localities might be newly infected with this most dreaded form of malarial infection.

Latent malarial infection complicating other diseases.—A very large number of latent malarial infections in Americans occur as complications of other disease processes, and it is most important that the malarial factor in such cases be recognized and removed.

As an illustration of the frequency with which latent malaria complicates other diseases I have compiled the following table, giving the original diagnosis in 106 cases in which a latent malarial infection was found to exist. In nearly all of these cases treatment with quinine, by removing the malarial infection, resulted in very marked improvement.

TABLE 1.

Diagnosis	No. of Cases	Diagnosis	No. of Cases
Chronic dysentery	15	Acute dementia	2
Chronic diarrhea	20	Convalescent from operation	1
Pulmonary tuberculosis	3	Arthritis deformans	1
Fractures and wounds	11	Retinitis	1
Chronic gastritis	8	Varicocele	1
Amebic dysentery	15	Tachycardia	1
Chronic indigestion	3	Uncinariasis	1
Hernia	3	Diabetes mellitus	1
Otitis media	3	Paraplegia	1
Acute melancholia	3	Acute endocarditis	1
Rheumatism	3	Hemorrhoids	1
Syphilis	2	Adenitis, cervical	1
Insanity	2		
Paralysis	2	Total	106

A consideration of some of the data given in the above table is of interest.

In 15 cases of chronic dysentery, non-amebic in type, a latent malarial infection was discovered. All of these patients gave a history of having suffered from malaria in the Philippines, but as not having had any symptoms of the disease since reaching the United States. The much-controverted statement that there exists a form of dysentery due to the localization of the malarial plasmodia in the capillaries of the intestine is of interest in connection with these cases, as treatment of the malarial infection with quinine resulted in every case in marked improvement and ultimately in recovery. From personal observation I am convinced that not infrequently a bloody diarrhea accompanies certain estivo-autumnal infections, when acute, and it is not at all improbable that cases may occur of chronic dysentery (so called) due to the invasion of the intestine by the plasmodia or to the action of a malarial toxin. The same remarks apply to the 20 cases of chronic diarrhea in which a coincident latent malarial infection was demonstrated.

In 15 cases of amebic dysentery a latent malarial infection was observed. In these cases *Entameba dysenteriae* was present in the feces and one of the species of plasmodia in the blood.

In nine cases of fracture and two of bolo wounds an examination of the blood showed the presence of malarial parasites, although no symptoms of infection were noticeable. This was also true in one case convalescent from an operation.

Latent infection in natives of the Philippine Islands.—The observations herein noted upon latent malarial infections in natives of the Philippines were made at Camp Stotsenburg, Pampanga Province, during a five-months' tour of duty there.

The subject of latent malarial infection in certain native races has been studied by many observers, notably Koch, Stephens and Christophers, and Annett, Dutton, and Elliot, in Africa, and James, in India.

Koch,[6] in West Africa, found that 100 per cent of native children under two years of age showed malarial parasites in their blood, and that the percentage of malarial infections decreased with advancing age; thus, children from two to five years of age showed 46 per cent infected and from five to 10 years of age, 23.5 per cent, while those over 10 years old were free from infection.

Stephens and Christophers,[7] at Accra, West Africa, found from 23 to 90 per cent of babies infected; up to eight years of age, 20 to 57 per cent; up to 12 years of age, 28 to 30 per cent, and after the twelfth year infection was found to be rare. At Lagos, of children under two years of age, 50 to 100 per cent were infected; from two to five years, 40 to 75 per cent, and from five to 10 years, 25 per cent. Annett, Dutton, and Elliot,[8] working in Nigeria, obtained the results given in the following tabulation.

TABLE 2.

Age of Child	Number Infected
0 to 1 year	27.3 per cent
1 " 2 years	63.0 "
2 " 3 "	63.0 "
3 " 4 "	51.0 "
4 " 5 "	48.8 "
5 " 6 "	34.8 "
6 " 7 "	6.6 "
7 " 8 "	27.5 "
8 " 9 "	25.0 "
9 " 10 "	14.0 "
10 years and above	10.0 "

Averages: 0 to 5 years, 51.8 per cent
5 to 10 years, 25.0 "
10 years 10.0 "

James[9] in India found that the percentage of malarial infection among native children varied very greatly in different localities, being zero in some places and as high as 86 per cent in others. Thus at Mian Mir, children up to three years of age showed 80 per cent infected; up to five years, 66 per cent; up to 10 years, 50 per cent, while from 10 years upward none were found infected. At Ennur, children up to three years of age showed 65 per cent infected; up to five years, 51 per cent; up to 10 years, 46 per cent, and up to 15 years, 16 per cent. He found no infection in natives over 15 years of age. He also states that in the most malarious localities the immunity of the adult is very apparent, but that in those localities in which the malarial ratio is low an immunity of the adult does not appear to be established. At the time of my observations at Camp Stotsenburg the conditions present were briefly as follows:

The post is situated upon the western border of the great Pampangan plain, in the foothills of the Zambales mountains, at a considerable elevation. The soil is of volcanic nature and becomes dry, even after the heaviest rains, in a few hours. So far as I could ascertain there were no breeding-places of *Anopheles* within one mile of the post, and as these insects were at all times present, it will be seen that the prevalent idea that a mosquito will fly but a short distance in search of food is disproved in this instance. There is a considerable area of jungle country about the station, some of it within a radius of a few hundred yards; but I failed, after the most careful exploration of these jungles, to find any breeding-places of *Anopheles* nearer to the post than one mile. It was invariably found that when the grass about the post was allowed to grow to any great length, mosquitoes and malaria increased; on the other hand, when it was cut, both mosquitoes and malaria diminished very appreciably. During the latter portion of the rainy season the *Anopheles* begin to appear in great numbers, and reach their maximum during the months of November and December. This coincides with the increase in malarial infections at this post, as is shown in the following table:

TABLE 3.

Month	No. Infections	Month	No. Infections
1904		1905—*Continued*	
August	24	April	10
September	57	May	14
October	24	June	17
November	58	July	11
December	75	August	29
1905		September	31
January	76	October	43
February	27	November	54
March	20	December	126

During the five months I was stationed at Camp Stotsenburg I observed 386 cases of malaria in which I was able to demonstrate the parasite in the blood. Of these, 248 occurred in Americans and 138 in natives. As regards the type of infection, 98 were infected with the tertian plasmodium, of which 63 were Americans and 35 natives; eight with the quartan, of which two were Americans and six natives; and 272 with the estivo-autumnal plasmodia, 183 being Americans and 89 natives. Of the estivo-autumnal infections, 258 were due to the tertian estivo-autumnal plasmodium and 14 to the quotidian variety. There were eight combined infections with the tertian and the estivo-autumnal tertian plasmodium. Of these cases there were 227 initial infections, all in Americans; 38 recurrent infections; in Americans, 18, and in natives, 20; and 115 latent infections, all in natives.

It appeared probable to me in considering the malarial situation at this post that the natives living in the barrios in close proximity were in all likelihood the principal source of infection, for while, as has been stated, the sanitary conditions in the post proper were such as to prohibit the belief that much malarial infection could originate there, breeding-places of mosquitoes abounded in the barrios and mosquitoes of the genus *Anopheles* were much more

numerous in them than at the post. The barrios were constantly visited by the soldiers, especially at night, and in them the conditions for the spread of both mosquito and human infection were ideal. In order to determine how large a percentage of the native population of these barrios was infected, I made blood examinations, whenever they could be obtained, of the natives living within two miles of the post. The results proved beyond question that the origin of malarial infection at Camp Stotsenburg was to be found very largely in the natives being in the immediate vicinity, and that any efforts to limit the disease must take this condition into account. In a considerable number of the cases of latent infection, even in the youngest children, a history of previous attacks of fever could be obtained, but in none of them were any symptoms of malaria observed at the time of examination. In all, the blood of 225 natives was examined and 115, or 51.1 per cent, were found to be infected. This percentage of latent malarial infections should not be regarded as typical of all localities in the Philippines, for many localities are free from malaria, while in others the malarial index is low. Routine blood examinations of the natives will give much valuable information as to the endemicity of malarial infection in these islands, and such examinations should be made before permanent military posts or residences are established in the Tropics.

Of the 115 infections found, the tertian plasmodium was present in 29, the quartan in six, and the estivo-autumnal in 77; of the latter, 73 were due to the tertian estivo-autumnal plasmodium and four to the quotidian. There were three infections with the tertian and the tertian-estivo plasmodium.

Latent infection in the adult native.—I was able to examine the blood of but 45 adults, of which 28, or 62.2 per cent, were infected; of these, five were due to the tertian plasmodium and 23 to the tertian estivo-autumnal plasmodium. It is very probable that a further study of the blood of a larger number of adults would materially reduce this great percentage of infections, but, from the results obtained, it is evident that the adult Filipino is more often infected than the negro in Africa or the natives of India.

Both Koch in Africa and James in India, found that the percentage of infections in adult natives is very small. In the planting districts of the Duaro, James found no adults with plasmodia in their blood, although 65 to 75 per cent of the children were found to be infected. It is probable that the adult Filipino possesses little or no immunity to malaria, despite the fact that in malarial localities such adults have from

childhood suffered from repeated attacks of the disease. I have notes on several native adults who, within two years, were admitted to the hospital from 8 to 16 times with malarial infection.

This lack of immunity to malaria in the adult native of this country appears to me to be a very significant and important economical fact, for it is invariably true that a people suffering from long-continued malarial infection are poor producers, especially along agricultural lines, where strength and endurance are demanded.

Latent infection in native children.—Of the 180 children whose blood was examined, 87, or 48.3 per cent, showed the presence of malarial plasmodia. Of these infections 34 were due to the tertian plasmodium, six to the quartan, and 44 to the estivo-autumnal variety; of the latter 40 were infected with the tertian estivo-autumnal plasmodium and four with the quotidian estivo-autumnal plasmodium. There were three infections combined with the tertian and the tertian estivo-autumnal plasmodium.

The infections in children diminished in number with advancing age; thus, between the ages of one month and five years, among 40 children, 79 per cent were infected; between five and 10 years, 37 per cent; and between 10 and 15 years 24.5 per cent. These results agree with those of Koch, Stephens and Christophers, James and others, who invariably found that the younger the child, the more susceptible it was to malaria.

The following table illustrates the relationship between malarial infection and the age of the individual, as observed in the barrios about Camp Stotsenburg; it gives the number examined, the number attacked in five-year periods, the percentage of those infected, and the type of infection.

TABLE 4.

Age	No. infected	Per Cent Infected	Tertian	Quartan	Estivo-Autumnal
1 to 5 years—40 children.....	30	72.5	10	4	16
5 to 10 " —54 "	20	37.0	8	1	10
10 to 15 " —53 "	13	24.5	5	1	7

Only 147 children are considered in the above table, as only in that number could the age be ascertained with certainty.

TABLE 5.

Barrio	Age	No. Examined*	No. Infected	Per Cent Infected
A	1 to 5 years	6	5	83.3
	5 to 10 "	5	3	60.0
	10 to 15 "	6	2	33.3
	Over 15 "	5	3	60.0
B	1 to 5 years	10	8	80.0
	5 to 10 "	7	4	57.1
	10 to 15 "	6	2	33.3
	Over 15 "	20	10	50.0
C.....	1 to 5 years	12	12	100.0
	5 to 10 "	6	5	83.3
	10 to 15 "	3	2	66.6
	Over 15 "	14	9	64.2

* In explanation of the small number of individuals examined between the various ages it should be stated that the barrios mentioned are simply collections of a few huts, each *barrio* not containing more than a dozen or so, with a total population each of from forty to sixty individuals.

Barrio infections.—It is interesting to observe how the little barrios, all situated within two miles of the post, varied as regards the number of infected individuals. The foregoing table well illustrates this point.

In the town of Mabalacat examinations were made of the school children with the following results:

Of children between the ages of five and 10 years, 25 were examined, of which seven, or 28 per cent, were infected; of children between 10 and 15 years of age, 35 were examined, of which seven, or 20 per cent, were infected.

In the town of Dolores, 11 school children between the ages of five and 10 years were examined, of which seven, or 63.6 per cent were infected, while of 13 children between 10 and 15 years of age, four or nearly 31 per cent, were infected.

In Mabalacat the children of the better class of natives attend one school while those of the lower class attend another, but the percentage of latent malarial infections was practically the same in both schools.

Family infection.—Not only do the barrios differ in the ratio of malarial individuals to those in health, but certain portions of the larger barrios are most malarious, while other portions are almost free from the infection; investigation always demonstrated that this is due entirely to the number of *Anopheles* breeding-places about such areas. Thus the malarial infections may be said to be strictly local infections, being confined within certain well-defined limits, even in infected localities.

Not only were certain portions of the barrios free from infection and badly infected in others, but it was observed that malaria was very largely a family disease, certain families suffering severely while others were free from infection. The following table illustrates the family character of malarial infection, being compiled from the data obtained in one barrio where all the families resided:

TABLE 6.

Family	No. Members	No. Infected	Variety
1	4	2	1 es.-autumnal; 1 tertian
2	3	2	2 estivo-autumnal
3	4	2	1 es.-autumnal; 1 tertian
4	5	4	2 es.-autumnal; 1 tertian 1 quartan
5	4	2	2 estivo-autumnal
6	3	2	2 estivo-autumnal
7	4	3	2 es.-autumnal; 1 tertian
8	3	2	1 es.-autumnal; 1 tertian
9	3	2	2 tertian
10	6	4	2 es.-autumnal; 2 tertian

In considering this table it should be remembered that these infections were latent in character, and I have repeatedly observed families in which every member was suffering from a malarial infection, either latent or active. Such are numbers four and 10 in the above table. Family No. 4 is of interest because, of its five members, one was suffering from a severe attack of tertian malaria at the time that I made the blood examination, while of the other four two presented estivo-autumnal, one the quartan, and the other the tertian plasmodium in the blood, so that in this one family all the varieties of the parasite could be studied.

Pathology of latent infections.—The study of the pathological lesions found in cases of latent malarial infection demonstrates that

the malarial plasmodia may unde go schizogony within the spleen without producing symptoms of the disease.

If we confine the term "latent infection" to those cases in which no symptoms are present and no parasites are found in the peripheral blood after repeated examinations, it will at once be seen why the pathology of this condition has not been thoroughly investigated. Such cases of malarial infection will only be discovered at autopsy, the patient having perished from some other disease. Outside of the Tropics and the more malarial regions of our own country, such cases are very rare. I have observed seven cases in which, at autopsy, death having been due to some other disease, latent malarial infection was discovered upon microscopical examination of the viscera. During life none of these patients presented any symptoms of malaria, nor were malarial parasites found in the blood after repeated examinations. The patients were under observation in the hospital for several weeks, were carefully studied clinically, and in none was malarial infection suspected. Of the seven cases three were benign tertian infections and four estivo-autumnal infections of the tertian type.

The pathological lesions found in the tertian cases were confined to the liver and spleen, especially the latter. The spleen was considerably enlarged, decreased in consistence, and somewhat pigmented. Microscopical examination of sections showed intense congestion of the sinuses together with pigmentation, the latter being marked along the edges of the Malpighian bodies and the fibrous trabeculae. Many of the cells of the splenic pulp were pigmented. Numerous parasite-infected red cells and pigmented leucocytes were present, and while the infected red cells were not nearly as numerous as in acute infections or as in more advanced latent infections, they were still quite numerous. The plasmodia were in about the same stage of development in the individual case, but it so happened that the patients had died at such a period that the entire cycle of the tertian parasite within the human body could be followed. As far as could be ascertained the plasmodia did not differ in their appearance from those seen within the red cells in the peripheral blood during an acute infection. The segmenting bodies were numerous in one case, the segments, in fresh smears from the spleen, appearing somewhat more refractive and clearly outlined than in the peripheral blood. The staining reactions of the plasmodia did not differ from those present in the blood.

The capillaries also contained pigmented leucocytes and macrophages, the latter containing much pigment in large blocks and often one or more parasites.

The liver, macroscopically, did not differ in appearance from that of the normal organ but upon microscopical examination the capillaries showed within them a few pigmented leucocytes, some containing what appeared to be degenerating plasmodia. No infected red cells were observed. In the estivo-autumnal infections the spleen

appeared as in the tertian infections, but the enlargement and pigmentation were not as marked. The consistence was much decreased. Upon microscopical examination the same changes were found, as in the tertian cases, the splenic sinuses being congested, the cells of the splenic pulp increased in number, considerable pigmentation was present, and the presence of infected red cells and melaniferous leucocytes was noted. The infected red cells were not as numerous as in the tertian infections. The parasites observed within the red cells were almost all in the same stage of development, but the four patients showed all stages of schizogony of the plasmodia, no single one of them, however, showing all the stages. The young forms of the estivo-autumnal parasite were similar in appearance to the young forms found in the peripheral blood, being small, hyalin rings, well defined, and presenting, in the fresh smear, marked ameboid motion of limited extent. The older parasites were round or ringlike, and contained a small amount of pigment in the form of very fine, reddish-brown granules. In one case numerous segmenting bodies were observed, the segmentation always taking place within the red cell. The segments varied in number, the largest number counted being 24, the smallest 12. No crescents were observed in any of the sections.

The pathological changes in the liver were similar to those in the liver of the tertian cases. No infected red cells were found, although a considerable number of pigmented leucocytes were observed, together with some free pigment.

The pathology of latent malarial infection may be summed up by the statement that before any clinical symptoms of infection are present the plasmodia are undergoing normal schizogony within the spleen, and can be demonstrated within that organ either by splenic puncture or at autopsy. The lesions produced are the same in character as occur in acute infections but, of course, less extensive.

Cause of latency.—Many theories have been advanced regarding the cause of latency in malarial infection, and these will be considered very briefly under the discussion of the etiology of recurrences.

Practical deductions.—From the data which have been given I believe that it is obvious that latent malarial infections are of much importance from both epidemiological and clinical standpoints; it is certainly true that latent infections are a great source of malarial disease, and in the Tropics, the latent infection of the native is undoubtedly the principal source of infection of the white man. This is well illustrated in the study of malarial infection at Camp Stotsenburg, and the same condition obtains, without doubt, in every malarial locality in these islands. The importance of an examination of the blood of the native population of any given locality cannot be overestimated in the fight against malaria. This is especially true in the Tropics, for only through the results of such examination can the endemic areas of malaria be found and guarded against. Only

to one who has attempted it can the almost hopeless task of exterminating mosquitoes in tropical regions be appreciated, and, in fact, in many such localities it is impossible of accomplishment. This being so, it is apparent that it is useless to expect to rid a locality of malaria from which the mosquitoes cannot be eliminated, unless the infection is first stopped among the natives; and in the Tropics it appears to me that the greatest hope of success in combating malaria lies in the distribution of quinine to the infected native. It would then make no difference how numerous *Anopheles* might be, they would be harmless if the native were rendered free from the plasmodia by the use of quinine.

2. RECURRENT MALARIAL INFECTION.

It is probably a fact that with very few exceptions (and these most vigorously treated with quinine), every primary malarial attack is followed by one or more relapses or recurrences. By recurrences I mean the appearance of symptoms due to the same group of parasites that caused the original infection, and not a reinfection by another group. So common are recurrences in malaria that a prevalent belief is that "once a victim of malaria, always a sufferer from the disease;" while this belief is unfounded, recurrences often do persist for months, and sometimes, although very rarely, for years. They are most common and persistent in the estivo-autumnal infections, as would be expected from the greater resistance of these infections to treatment.

To the clinician the time elapsing between the initial attack and the recurrences is of greatest interest, while to the investigator the etiology of recurrences and the *modus operandi* have proven a fruitful field for contoversy, and, from the very nature of the problem, of theoretical deduction.

Time of recurrence.—Authorities differ somewhat as to the time of recurrence in the different types of malarial infection, and when we consider the difficulty of ruling out reinfections, especially in those who reside in a malarial locality, as well as the influence which treatment has in delaying recurrence, the slight difference in the time as given by various observers is indeed surprising. In malarious regions it is obviously impossible, in many instances, to be sure that

the reappearance of symptoms is not due to a reinfection, unless a different type of parasite be present than that found during the initial attack. As a basis for the computation of the time of recurrence Celli[10] reckons "as recurrent every case of fever which repeats itself in the same individual from the July of one year to the end of June of the following year, or during all the cycle of the same yearly epidemic." While, of course, errors are bound to occur with this method of computing recurrences, Celli believes, and I think justly, that for practical purposes it is as perfect as is possible under the circumstances. I have used this method in compiling the table of recurrences which follows, but have taken the further precaution of selecting only those cases which I am reasonably sure could not have become reinfected.

Borius,[11] an investigator working in Senegal, found that recurrences took place preferably upon the 7th, 14th, 21st, and 28th days after the initial attack. Of 226 cases, 128 relapsed, and of these 18 relapsed upon the 7th day, 64 on the 14th; 31 on the 21st; and 9 on the 28th day. Ninety-eight cases relapsed irregularly on the 9th, 10th, 16th, and 20th days. All of the cases took quinine at the time of the attack. Barudel[12] from his observations, concludes that quotidian fever most frequently relapses upon the 7th day, tertian upon the 14th, and quartan upon the 20th. Mariotti-Bianchi[13] concludes that benign tertian infections relapse between the 5th and 18th days after infection, while estivo-autumnal relapses between 5 and 21 days, but frequently between 5 and 9 days; Ziemann[14] found that in West Africa estivo-autumnal infections relapsed between 9 and 12 days after the primary attack, which, as he points out, is practically the same as the incubation period. Werlhof,[15] from his experience decides that tertian infections relapse most frequently in the second, and quartan in the third week, while Duden[16] claims that on the east coast of Africa, quotidian fever relapses almost invariably upon the seventh day.

Authentic recurrences after long intervals of time are rare, but unquestionably occur. Thayer[17] relates an interesting example and Mariotti-Bianchi observed in tertian infections recurrences between three and 13 months and in estivo-autumnal fevers, between four and eight months apart. V. Leyden[18] observed a recurrence of a malarial infection three years after the initial attack, and Schilling[19] has observed recurrences after eight and one-half months and after two and one-half years. As regards recurrences after such long intervals as two or three years I agree with Mannaberg[20] that the evidence is not sufficient to prove undeniably that the so-called recurrences were not reinfections and such cases appear very doubtful.

The following tables of recurrences in estivo-autumnal and tertian malaria are prepared from carefully selected cases in which reinfection was considered at least very improbable, and which I believe may be considered as portraying the exact length of time occurring between the relapses in these types of the malarial fevers.

The patients were all American soldiers observed in hospital and treated thoroughly with quinine during the active symptoms, while most of them received prophylactic doses of the drug once a week between the relapses, which undoubtedly delayed them somewhat.

TABLE 7.

ESTIVO-AUTUMNAL TERTIAN RECURRENCES.—TIME OF THE VARIOUS RECURRENCES IN 55 CASES OF TERTIAN ESTIVO-AUTUMNAL INFECTION.

Case No.	Date of Initial Attack	First Recurrence	Second Recurrence	Third Recurrence	Fourth Recurrence	Fifth Recurrence
1	Oct. 12	10 days	30 days	36 days	.....	.....
2	Nov. 19	12 "	.....	.....	.....	.....
3	Feb. 27	15 "	20 days	30 days	.....	.....
4	Nov. 2	18 "	30 "	30 "	.....	.....
5	Mar. 30	19 "	20 "	.....	.....	.....
6	Dec. 8	19 "	.....	.....	.....	.....
7	Jan. 24	20 "	.....	.....	.....	.....
8	Feb. 12	20 "	60 days	.....	.....	.....
9	Dec. 24	20 "	.....	.....	.....	.....
10	Feb. 6	20 "	20 days	.....	.....	.....
11	Feb. 6	20 "	48 "	.....	.....	.....
12	Dec. 25	21 "	33 "	.....	.....	.....
13	Mar. 1	22 "	.....	.....	.....	.....
14	Nov. 29	22 "	.....	.....	.....	.....
15	Nov. 14	24 "	.....	.....	.....	.....
16	Feb. 4	24 "	20 days	38 days	30 days	.....
*16½	Oct. 30	24 "	16 "	.....	.....	.....
17	Aug. 29	24 "	26 "	.....	.....	.....
18	Mar. 17	24 "	.....	.....	.....	.....
19	Feb. 4	25 "	16 days	20 days	.....	.....
20	Dec. 30	26 "	36 "	30 "	90 days	30 days
21	Jan. 26	26 "	48 "	90 "	.....	.....
22	Jan. 11	26 "	22 "	.....	.....	.....
23	Oct. 2	27 "	.....	.....	.....	.....
24	Nov. 2	27 "	.....	.....	.....	.....
25	Mar. 2	27 "	52 days	.....	.....	.....
26	Feb. 5	28 "	21 "	20 days	21 days	.....
27	Dec. 12	28 "	28 "	.....	.....	.....
28	Oct. 29	29 "	48 "	15 days	.....	.....
29	Jan. 17	30 "	.....	.....	.....	.....
30	Jan. 1	30 "	30 days	.....	.....	.....
31	Jan. 19	30 "	.....	.....	.....	.....
32	Jan. 20	32 "	.....	.....	.....	.....
33	Oct. 19	33 "	26 days	90 days	.....	.....
34	Jan. 19	34 "	40 "	.....	.....	.....
35	Oct. 18	34 "	50 "	.....	.....	.....
36	Jan. 25	34 "	26 "	17 days	.....	.....
37	Oct. 21	36 "	56 "	.....	.....	.....
38	Feb. 30	36 "	66 "	.....	.....	.....
39	Aug. 13	36 "	35 "	.....	.....	.....
40	Nov. 27	36 "	.....	.....	.....	.....
41	Sept. 1	37 "	49 days	.....	.....	.....
42	Oct. 18	38 "	.....	.....	.....	.....
43	Oct. 17	38 "	.....	.....	.....	.....
44	Aug. 13	38 "	.....	.....	.....	.....
45	Sept. 6	41 "	.....	.....	.....	.....
46	Oct. 31	42 "	20 days	.....	.....	.....
47	Jan. 1	45 "	30 "	.....	.....	.....
48	Nov. 3	46 "	21 "	.....	.....	.....
49	Dec. 7	49 "	.....	.....	.....	.....
50	Feb. 24	50 "	24 days	41 days	.....	.....
51	Oct. 24	51 "	39 "	.....	.....	.....
52	Jan. 18	61 "	156 "	.....	.....	.....
53	June 14	64 "	66 "	14 days	20 days	20 days
54	Mar. 3	80 "	120 "	96 "	.....	.....

*The case numbered 16½ brings the total of the table to 55 cases.

A consideration of the table shows that the first relapse in estivo-autumnal tertian infection occurred at periods varying from 10 up to 80 days; in none of the cases did a

relapse occur before 10 days after the initial infection, which does not agree with the results obtained by Mariotti Bianchi and Ziemann, already mentioned, who found that this type of malarial infection relapsed most frequently in from 5 to 20, and 9 to 12 days respectively. The administration of quinine probably delayed the relapse in my cases, but even so, it will be noticed that the vast majority of the cases did not recur until after the 24th day. Taken in periods of 10 days, an analysis of the table shows that six cases recurred between the 10th and the 20th days; 23 between the 20th and 30th days; 16 between the 30th and 40th days; five between the 40th and 50th days; and the remainder at periods later than the 50th day. The greatest number of relapses occurred between the 20th and 30th days, namely, 23, and almost as many between the 30th and 40th days, namely 16. Of single days five cases recurred upon the 20th day; five upon the 24th day; four upon the 36th day, and three upon the 26th, 27th, 30th, 34th, and 38th days.

In most instances secondary relapses occurred at longer intervals than the primary one, although numerous exceptions to this statement will be noticed in the table.

The statement that the longer the infection lasts the longer becomes the period between relapses is not borne out in this table, if the majority of the cases be taken into account. Thus in Case 16, relapses occurred at periods of 20, 38, and 30 days; in Case 20, at periods of 26, 36, 30, 90, and 30 days; in Case 26, at periods of 28, 21,20, and 21 days; in Case 36, at periods of 26, 34, and 17 days; in Case 50 at periods of 50, 24, and 41 days, and in Case 53, at periods of 64, 66, 14, 30, and 20 days.

Of the 55 cases, 36 had two relapses; 14, three relapses; 4, four relapses, and 1, five relapses; this well illustrates the persistency of estivo-autumnal infection, and its resistance to treatment, unless the treatment be continued for a long period of time.

A most interesting feature of the estivo-autumnal cases is the fact that 21 of them suffered from at least two relapses when the primary relapse occurred 27 days after the acute initial attack. In the benign tertian infections not a single secondary relapse occurred when the primary relapse occurred after the 26th day. In the estivo-autumnal infections it will also be noticed that no security against future recurrences is apparent when the primary relapse occurs after a long period of time from the acute attack, whereas in the benign tertian infections one can almost rest assured that secondary relapses will not occur if the primary relapse occurs a month after the acute initial attack.

Tertian recurrences.—The following table gives the data concerning recurrences in 18 cases of benign tertian malaria. The number is small, but while my records contain data covering hundreds of tertian cases, in only 18 cases can I be sure of genuine recurrence. The vast majority of tertian cases, if properly treated, do not recur, and very many of them recover spontaneously, although in such cases (which comprise, of course, only a very small number in my records of hospital cases) recurrence is much more common and, indeed, may be stated to be the rule.

A consideration of this table shows that tertian relapses occurred at periods of from 16 to 41 days; the shortest period after the acute initial attack was 16 days, while Mariotti-Bianchi found that recurrences in benign tertian appeared as early as five days and not later than 18. Of the 18 cases, relapse occurred in 1 in 16 days; in 1 in 18 days, in 1 in 19 days; in 2 in 20 days; in 3 in 21 days; in 2 in 22 days; in 1 in 27 days; in 3 in 30 days; in 1 in 33 days; in 1 in 37 days; in 1 in 38 days, and in 1 in 41 days. Secondary relapses occurred in 10 cases; a third relapse occurred in six cases; a fourth in three cases and a fifth in one case. No secondary relapse

TABLE 8.

TIME OF RECURRENCES IN 18 CASES OF TERTIAN INFECTION.

Case No.	Date of Initial Attack	First Recurrence	Second Recurrence	Third Recurrence	Fourth Recurrence	Fifth Recurrence
1	Nov. 2	20 days	21 days	.....	.....	.....
2	Aug. 4	18 "	20 "	.....	.....	.....
3	Aug. 28	19 "	30 "	26 days	46 days	.....
4	Nov. 6	20 "	24 "	.....	.....	.....
5	Jan. 17	20 "	32 "	30 days	24 days	.....
6	Nov. 23	21 "	20 "	26 "	.....	.....
7	Oct. 6	21 "	30 "	.....	.....	.....
8	Sept. 17	21 "	22 "	27 days	.....	.....
9	Aug. 27	22 "	36 "	.....	.....	.....
10	Feb. 12	22 "	18 "	16 days	27 days	30 days
11	Jan. 17	27 "	.....	.....	.....	.....
12	July 20	30 "	.....	.....	.....	.....
13	May 3	30 "	.....	.....	.....	.....
14	Nov. 1	30 "	.....	.....	.....	.....
15	Sept. 22	33 "	.....	.....	.....	.....
16	Sept. 1	37 "	.....	.....	.....	.....
17	Dec. 13	38 "	.....	.....	.....	.....
18	Sept. 22	41 "	.....	.....	.....	.....

occurred if the primary relapse occurred more than 22 days after the initial attack, and therefore, in tertian infections we may say that if a relapse does not occur within a month after the acute attack there is practically no danger of future recurrences.

A study of the two tables given enables us to conclude that in estivo-autumnal tertian infections, relapses occur most frequently between the 20th and 40th days after the initial acute attack, and in benign tertian infections between the 15th and 22d days; during these "periods of relapse," as they may be termed, quinine should be given in full therapeutic doses, and increased to the point of cinchonism at the appearance of the first symptoms indicative of a malarial attack. It should be remembered that quinine was given in prophylactic doses, once a week, to all the cases quoted in the tables, and thus the occurrence of the relapses was undoubtedly delayed somewhat, which accounts for the difference in my results and those of others who have described relapses in cases uninfluenced by quinine.

Etiology of recurrences and latency.—The etiology of recurrent and latent malarial infection should be considered together, as an explanation of the one would necessarily explain the other, since the infection must be latent in the system during the period between the recurrences. Many investigators have attempted to explain recurrence in malarial infection, but almost all of our knowledge of these subjects is theoretical and incapable of proof. It is evident that the plasmodia must exist in some form in the body during the intervals

in which no symptoms are present, and I have already shown, in considering the pathology of latent infection, that plasmodia may be demonstrated in the spleen of patients who have died from some other disease, and in whom no symptoms of malarial infection were ever present; these plasmodia did not differ in appearance from those observed in the peripheral blood in the acute malarial attacks, and, furthermore, were undergoing normal schizogony within that organ, but in numbers insufficient to produce clinical symptoms. In such cases it may be urged that after a certain period of multiplication the plasmodia become numerous enough to produce symptoms of malarial infection, and thus a relapse follows. While this is undoubtedly true in some instances, it will hardly account for long-interval relapses, for it is impossible to conceive that the plasmodia of malaria can continue to grow and multiply for weeks and months without becoming numerous enough to produce symptoms of infection.

To overcome this objection, Bignami[21] considers that the plasmodium exists in some latent form, perhaps encapsulated, in the spleen or other internal organ, which may not be rendered visible with our present staining methods, and which, resting as a spore, is only set free under certain favorable conditions, the nature of which we are ignorant of.

Celli, in discussing this subject, says: "How are these recurrences explained? It is difficult to say; perhaps they depend on forms resulting from sexual multiplication, that remain inert in some viscera—possibly the bone marrow—and, from time to time, invading anew the blood, give rise to new generations of the asexual cycle."

A. Plehn[22] interprets the basophilic granules, so commonly seen in the infected red cells in tertian malaria, as latent forms of plasmodia which multiply in the blood until conditions are favorable for the development of the large ameboid forms which produce the symptoms of infection. It is unnecessary to state that this explanation of basophilic granulation is wholly unique and untenable.

Recently Schaudinn,[23] in an excellent study of *Plasmodium vivax*, stated that recurrences are due to parthenogenesis of the marcogametes which are not fertilized by the microgametocytes; these remain in the human host and eventually liberate schizonts which penetrate the erythrocytes, undergo schizogony, and thus produce a

relapse. This process is completed in from 9 to 12 days, and, according to Ziemann and Mariotti-Bianchi, agrees with the period in which relapses most frequently occur. So far as I know Schaudinn's results have not been confirmed and it is difficult to understand how such a process explains relapses occurring at irregular intervals as shown in the tables given, where it is obvious that no regularity is present in the development of either the primary relapse or those succeeding it. How can the parthenogenesis of the macrogamete, which must occur in a cyclical manner, be the cause of relapses occurring all the way from 16 to 80 days after the initial attack of fever? For this reason, I am inclined to believe with Bignami and Celli that the cause of relapse will be found in a resistant form of the plasmodium (a latent form), which is capable of remaining unchanged within the human body for considerable periods of time, and which, under favorable conditions, undergoes further development and gives rise to the symptoms which are the evidence of a recurrence.

In considering the significance of intracorpuscular conjugation in the malarial plasmodia I shall discuss this subject further, indicating how this peculiar process may bear a very important relation to latent and recurrent malarial infection.

The significance of intracorpuscular conjugation in the malarial plasmodia.—In December, 1905, I described[24] a process of conjugation in the malarial plasmodia taking place within the infected red cells. This process had been described before by Ewing,[25] but my conclusions regarding its significance differed greatly from his, as will be seen in the following brief summary of his important work upon this subject. Ewing considered the process of rare occurrence and of comparatively little significance. In describing it he says:

> In four cases of tertian infection I have encountered appearances which seem to admit of no other explanation than that of conjugation of malarial parasites. In a considerable number of other cases similar appearances were found, but much less frequently. A great many red cells showed double infection with young rings. In many instances these rings were entirely separate, each exhibiting a singular large granule of chromatin. Many cells, however, contained two rings which were clearly fused together along one segment of the ring, and two large chromatin granules were then invariably found at different poles of the rings.

Describing what he considers as a later stage of the same process, he says:

On examining the parasites in later stages of development, most of them were found to have lost the ring form, and to have spread out into a large number of threads, with nodal thickenings, variously curled in the red cell. These threads evidently represented the pseudopodia of a very active ameboid stage. The chromatin masses were now subdivided into 10 or 12 granules, but in the majority of the cases the masses were far apart, and showed no tendency to unite. In many cells, however, the ameboid figures were less marked, and the masses of chromatin lay side by side, united by a little achromatic substance.

Regarding the significance of the process Ewing is undecided. He says:

It would seem that a process so fundamental as the conjugation of individuals, if it occurs at all, ought to be an invariable feature of every active infection, but there is not sufficient evidence upon which to base any such claim. The four cases referred to as furnishing numerous clear examples of conjugation were selected on account of the abundance of the conjugating forms, but in many other cases less numerous, though equally distinct, examples were seen, indicating that the process is of very frequent occurrence. On the other hand, it must be admitted that the majority of specimens from routine cases fail to show any distinct traces of the process; from which it may be concluded that conjugation is not an essential feature of the growth of the parasite.

In a later contribution he says:

The extent and significance of this form of conjugation it is difficult to determine. In the cases in which it can be profitably studied, parasites are very abundant, and in most cases few indications of the process can be detected. It is, therefore, probably not essential to sporulation, and when parasites are scanty the chances of finding typical examples of conjugating pairs are greatly reduced, but the peripheral blood may not be a complete index of the process in the internal viscera. It seems probable that conjugation occurs in the first generations of the infection, and becomes less frequent as the disease progresses, the infection in the human host thereby tending to limit itself.

The material for my own studies upon this subject consisted of nearly 300 cases of malaria presenting clinical symptoms, observed at the U. S. Army General Hospital at the Presidio of San Francisco, Cal., together with over 100 cases of latent infection observed at the same place, a series of 75 latent cases in native Philippine children studied at Camp Stotsenburg, and 96 cases of acute infection observed at that post and in Manila. As a result of my studies, which cover a period of over five years, I am convinced that intracorpuscular conjugation is not an accidental occurrence of no essential importance in the life-history of the malarial plasmodia, but is a process which is most essential and one that occurs invariably in all acute infections uninfluenced by quinine and also in all recurrent cases in which the infection is of sufficient strength to produce marked clinical symptoms.

Morphology of intracorpuscular conjugation.—The process may be roughly divided into three stages. In the first stage, or stage of protoplasmic union, the two young hyalin rings are in contact, and careful examination demonstrates that at the point of contact there is a direct union of the protoplasm; in stained specimens it will be noted that the chromatin of the nuclei of the two parasites is separated and that the union begins in the protoplasm of the plasmodia; in fact, it should be stated that intracorpuscular conjugation always occurs between two young hyalin ring forms and is always completed before the formation of pigment. In no case can conjugation be recognized after the formation of pigment, and the large pigmented bodies described by Ewing as conjugating bodies in which the chromatin had divided into several distinct masses are easily recognized to be normal presegmenting plasmodia, in which such a division of the chromatin is always present. In this stage the chromatin masses may be situated at any portion of the periphery of the two rings, but it is extremely rarely that they lie in apposition at this stage. I have been unable to detect any differences in the appearance of the two conjugating bodies, for while one may occasionally be a little larger than the other, this is not so as a rule, and the chromatin masses are always of the same size.

In the second stage, which may be designated as that of complete protoplasmic union, the chromatin masses become situated in the protoplasm of one organism, formed by the gradual union of the protoplasm of the two; the chromatin granules may be opposite one another or at any portion of the periphery of the plasmodia, sometimes almost in apposition. The complete union of the protoplasm of the two plasmodia results in a more or less perfect ring-shaped organism, slightly larger than either of the original parasites, containing two masses of chromatin, surrounded by achromatic substance.

The third stage of the process, or stage of chromatic union, is characterized by the union of the chromatic granules, with the apparent exclusion, in many cases, of a very minute grain of chromatin, before the union is complete.

Briefly stated, then, intracorpuscular conjugation consists in the complete and permanent union of the protoplasm and nucleus of

two young amebula within the erythrocyte. It is absolutely necessary to the maintenance of malarial infection in man, and in these instances in which it does not occur, the plasmodia undergo a sexual sporulation for a limited time and then perish, thus leading to spontaneous recovery. It is present most typically in those cases in which the clinical symptoms are most severe, and is present in all the varieties of malarial infection, although most easily observed in the estivo-autumnal infections.

Conjugation in Protozoa in general and its significance.—In order to understand the significance of intracorpuscular conjugation as seen in the malarial plasmodia, it is necessary to review briefly the phenomena and significance of conjugation as they occur in other protozoan organisms, for it is only by thus summing-up, as it were, the significance of the process in all the forms of the Protozoa in which it has been observed, that we are enabled to arrive at any conclusions regarding its significance in the plasmodia of malaria.

The process of conjugation is common among the Protozoa and may be observed in all its gradations from the union of absolutely similar organisms, in which sex cannot be distinguished, to the union of highly specialized forms in which the male and female organisms may be easily distinguished.

The Rhizopoda.—Conjugation occurs comparatively seldom in the Rhizopoda most frequently among the Amebina and Helioza. Among the Amebina conjugation is probably much more common than is generally supposed. Holman[26] observed the temporary union of a large and small ameba, resulting apparently in the production of swarm-spores in each. Penard[27] describes the union of different-sized individuals in *Amoeba spatula*, the larger absorbing the smaller, the union being permanent. I have observed conjugation in *Entamoeba coli* several times, the process consisting in the permanent fusion of apparently similar individuals, the protoplasm of the two organisms first blending, followed by the fusion of the nuclei. In *Entamoeba dysenteriae* (*histolytica*) I have observed conjugation frequently, and I believe that the process is of greater import in the life-history of these parasites than is generally thought.

Among the Heliozoa, conjugation has been certainly demonstrated in *Actinophrys sol* and in *Actinosphaerium Eichornii* by Schaudinn;[28] in the former free-swimming forms unite, coalesce, and develop a firm protective covering; the union is protoplasmic, the nuclei remaining distinct, and finally divide, thus making four nuclei; two of these fuse, and give rise by mitosis to daughter-cysts, while the remaining two degenerate and disappear. In *Actinosphaerium Eichornii* conjugation is stated to be common before reproduction commences.

The Flagellata.—Among the Flagellata conjugation is very common and may occur between similar-sized individuals, or between individuals dissimilar in appearance. The product of the conjugation or the *zygote* gives birth to the motile organisms which reproduce without conjugation. To Dallinger and Drysdale[29] we are indebted for much of our knowledge regarding conjugation among the monads.

In *Monas Dallingeri* (*Cercomonas crassicauda*) reproduction occurs by fission of the free-swimming forms and by the conjugation of ameba-like forms, which upon coming in contact immediately coalesce; this "resting stage" persists for about six

hours, when the organism ruptures and liberates multitudes of minute spores, which develop into the adult organism and reproduce by fission as before. In *Oikomonas Dallingeri* certain of the organisms do not proceed to fission, but undergo changes in morphology consisting of the withdrawal of the flagella and great increase in the size of the nucleus; these bodies coming in contact with adult individuals, fuse with them, and become encysted; after from four to five hours the cyst ruptures and very minute spores are liberated which develop into the ordinary dividing forms. In *Heteromita uncinata* the conjugating organisms are differentiated by their size, larger and smaller individuals being seen, the former possessing a contractile vesicle at the anterior end; conjugation consists in the absorption of the smaller form by the larger, followed by encystment, and finally by the rupture of the cyst and the liberation of spores which become the ordinary dividing forms.

I have repeatedly observed what were undoubtedly conjugating forms in *Trichomonas* and *Cercomonas intestinalis* as well as in *Lamblia intestinalis*. In these species conjugation was preceded by the withdrawal or loss of the flagella and the development of irregular or round ameba-like forms; contact of two such organisms resulted in complete fusion and the production of a cystlike body which appeared clear and hyalin. I have not been so fortunate as to observe the rupture of the cyst and the liberation of the young spores.

Pringsheim[30] describes the process of conjugation in *Pandorina* as occurring between two individuals of the swarm-spores, which meet and fuse; the resulting organism encysts and develops within the cyst a swarm-spore, which is liberated when the cyst is placed in a favorable environment; the liberated swarm-spores divide and eventually form the sixteen-celled *Pandorina* colony.

The Sporozoa.—Among the Sporozoa the process of conjugation is highly developed and becomes largely a sexual phenomenon, the male and female organisms being, in most instances, easily distinguishable. However, conjugation in this class of organisms may be very simple, as has already been shown in the description of intracorpuscular conjugation in the malarial plasmodium, where no sex differentiation can be demonstrated.

In the *Gregarinida, Coccidiida,* and *Haemosporidia* conjugation occurs and has been extensively studied by Schaudinn and Siedlecki,[31] Wolters,[32] Grassi, and Feletti,[33] Celli and Sanfelice,[34] MacCallum,[35] and Ross.[36]

Gregarinida.—In this order sporulation is generally preceded by the fission of two gregarines and their encystment. Wolters in *Monocystis agilis* describes the process of conjugation as consisting of the following phenomena: apposition of the end of each organism; mitosis of each nucleus, one portion of each being extruded, the other two daughter-nuclei fusing through an opening in the wall of the organisms at the point of apposition; mitosis of the fused nucleus, one daughter nucleus then going to each conjugant; mitosis of each daughter-nucleus and the formation of spores. This method of conjugation has not been confirmed.

In those instances in which two gregarines unite and become encysted each nucleus reduces, a large portion of it disappears, and, as shown by Siedlecki,[37] a new nucleus, is formed which divides by mitosis; the daughter-nuclei now divide and the division is repeated until the cyst is filled with many nuclei. Each nucleus is surrounded by a portion of the protoplasm and thus *gametes* are formed which use and produce *sporozoites* or the infective organism.

Coccidiida.—Schuberg[38] was the first to suggest conjugation in the *Coccidiida* and Schaudinn and Siedlecki the first to demonstrate that this process is a developmental necessity in these organisms, is a true sexual phenomenon, and results in the fertilization of the female organism. In the *Coccidiida* sexual dimorphism exists, and an alternation of generations occurs, one asexual and the other sexual. In the asexual cycle the coccidia are intracellular in the host, where they sporulate, and the *merozoites*, leaving the host cells, invade new ones; in the sexual cycle conjugation occurs between specialized organisms, the male, or *microgamete*, and the female, or *macrogamete*, and sporogeny follows. Conjugation occurs within the infected individual, but is extracellular.

Hemosporidia.—It is now well known that sexual conjugation occurs among the *Hemosporidia* of both animals and man. The plasmodia of malaria, for example, undergo an asexual cycle of development in the blood of man and a sexual cycle in the body of the mosquito. In the latter, conjugation is absolutely essential to sporulation, but here, in contradistinction to the intracorpuscular conjugation which occurs in the blood of man, the conjugating organisms are sexually differentiated, and in addition intracorpuscular conjugation is not essential to sporulation, but only to the continuation of the infection. In the mosquito conjugation is a fertilizing process *per se*, while intracorpuscular conjugation simply stimulates the organism to renewed activity; the first is a true sexual process, the latter an asexual one, and thus in the two cycles of the malarial plasmodia conjugation is illustrated in its simplest and most highly developed phases.

As conjugation of the malarial plasmodia in the mosquito is so well known I shall not describe it further.

In *Lankestrella* and *Karyolysus*, hemosporidian forms inhabiting cold-blooded vertebrates, Labbe[39] has described a process of conjugation which approaches very closely to intracorpuscular conjugation in the malarial plasmodia. A *trophozoite* after developing for a while in a blood corpuscle becomes free and conjugates with a similar form; the *zygote* thus produced may penetrate a second blood corpuscle or may invade a cell of the spleen, kidney, or bone-marrow, where it becomes encysted; in the cyst are developed *sporozoites* which are liberated when conditions are favorable.

The Infusoria.—It was in this class of the Protozoa that conjugation was first correctly interpreted by Bütschli[40] and Englemann,[41] and here it occurs almost universally. In no other class of the Protozoa is the truth of the statement that the process is one of "rejuvenation" so well illustrated. Reproduction normally occurs in this class by binary division, or less often by multiple division after encystment; whatever the method, reproductive activity finally ceases and conjugation then occurs, restoring the vital activities of the cells by bringing about a regeneration of the protoplasm, and especially of the nucleus. Maupas[42] and Hertwig,[43] as well as Bütschli, have demonstrated that when conjugation occurs, the micronucleus and the macronucleus become separated. The micronucleus divides twice, forming four daughter-nuclei in each of the conjugating individuals; of these eight daughter-nuclei, six degenerate, while the remaining two fuse, the macronucleus meanwhile undergoing complete degeneration. After the formation of the new nucleus it divides and a portion of it forms a new macronucleus. As a rule the conjugating organisms separate, but the union may be permanent.

In *Paramecium aurelia*, Balbiani[44] demonstrated that conjugation occurred after

a series of generations had been evolved by ordinary transverse division, and that it lasted for five or six days, during which time important changes occurred within the conjugants, which finally separated and gave rise to individuals capable of developing as usual.

Gourvitch,[45] Strong,[46] and others have described conjugation as occurring in *Balantidium coli*. Gourvitch states that the conjugating pairs unite and form oval cysts, but his work has not been confirmed and it is probable that the process described by Strong, i. e., the simple fusion of adult organisms is what really occurs. In all probability, after a certain period of time, the conjugants separate and reproduce as before.

General significance of the process of conjugation in the Protozoa.— The process of conjugation in the Protozoa was first observed and described by O. F. Müller.[47] His work was confirmed by Balbiani, who claimed that certain of the Protozoa not only reproduced by simple division but also by conjugation, the latter being a sexual act leading to the formation of the young parasites. It is to Bütschli that we owe the correct interpretation of conjugation in the Protozoa. He observed that continued reproduction of many of these organisms by simple division led eventually to the exhaustion of the capability of division, and thus to the death of the organisms. He, therefore, regarded the process of conjugation as intended to bring about rejuvenescence of the nearly exhausted individuals of a generation of organisms. Englemann confirmed the interpretation of Bütschli, and thus defines the process: "The conjugation of the infusoria does not lead to reproduction through 'eggs,' 'embryonic spores,' or any other kind of germ, but to a peculiar developmental process in the conjugating individual, which may be designated as reorganization." In other words conjugation is intended to bring about a restoration to former reproductive activity, this result being secured by a rejuvenescence of the vital activities of the organism.

Calkins,[48] to whose admirable work I am indebted for many of the references in this contribution, in his discussion of the subject says:

If, as Minot suggested, every newly born organism be regarded as having a certain initial potential energy which is gradually used up in its life-activities to be restored by conjugation, then the union of two cells may be interpreted as a renewal of vigor or a rejuvenescence. . . . . The force of these views as to the need of conjugation for different species of infusoria, at least, can hardly be questioned, for, as repeatedly stated in the previous chapters, reproduction by simple division may go on for a certain number of generations, but cannot continue indefinitely, unless at certain intervals, which Maupas

has shown to be more or less definite, two individuals unite in conjugation. This union, in some wholly unexplained way, imparts to each of the conjugants a renewed vitality, or, in Bütschli's words, a renewal of youth, expressed by increased activity in movements and reproduction. Conjugation thus, as R. Hertwig insists, is not the beginning of a series of reproductive acts, but occurs at or near the end of such a series. . . . . The phenomena of the so-called sexual reproduction and sex differentiation have, in all probability, grown out of this apparently fundamental requirement of living protoplasm, namely, the periodic union of two cells.

Again he says:

The various conjugation phenomena seen in the Protozoa seem to show that each cycle starts with a certain potential of vitality which is gradually exhausted in the vegetative activities of the long line of individuals formed by simple division, or by spore formation.

That conjugation is not a reproductive act in many of the Protozoa is shown by the fact that the time consumed in conjugation is sufficient for reproduction by simple division to occur many times; this is well illustrated in those Protozoa in which a resting stage succeeds conjugation.

While fertilization is generally the result of conjugation it does not follow that the act is a reproductive one, for, as has been pointed out by Hertwig, "a reproductive process is bound up with the encystment of *Actinosphaerium*, whereby the mother-cyst gives rise to many primary cysts, each primary cyst to a germ-sphere, each germ-sphere to new individuals. Reproduction here precedes fertilization and the latter has no effect upon the former."

In reviewing the phenomena of conjugation in the Protozoa it has been shown that in many of them, after the union of the conjugants, a resting or *zygote* stage succeeded, in which the organisms possessed greater resistance to injurious influences, and in which they remained latent, so to speak, and no further development occurred until conditions were favorable. This is true in almost all the lowly forms of plant life in which conjugation has been observed and in many of the Protozoa. A most typical example is found in *Pandorina morum*, in which the *gametes* conjugate and assume a resting form, which is encysted; under favorable conditions the cyst ruptures and one or more individuals emerge and, dividing, in time the typical colony is again formed.

The conjugation of a protozoan within the body of its intermediate host is purely sexual in its nature and is followed at once by repro-

duction. Such a type of conjugation should be clearly distinguished from that in which there is a union of two individuals followed by a period of inactivity or a *zygote* stage. The sexual type occurring within an intermediate host is well illustrated by the union of the *macrogametes* and *microgametocytes* of the malarial plasmodia within the mosquito, while intracorpuscular conjugation of the same organisms is a typical example of asexual conjugation. It is apparent, therefore, that asexual conjugation is not a reproductive act, but one intended to preserve the function of reproduction in a race threatened with extinction by repeated division, or, perhaps, intended to evolve a resistant form when conditions are unfavorable for reproduction in the ordinary way. Again to quote Calkins:

> From all the facts shown at the present time, the only conclusion that can be drawn is that conjugation, apparently, is not the cause of reproduction, but as Butschli, Englemann, and Minot long since pointed out, in some unknown way provides the energy for continuing the functions of the individual, including the power of reproducing.

Significance of intracorpuscular conjugation and its relation to latent and recurrent malarial infections.—Having thus briefly reviewed the process of conjugation and its significance as it occurs in Protozoa in general, we are in a position to consider the significance of that peculiar form of conjugation in the malarial plasmodia which I have called intracorpuscular conjugation. The conjugation of malarial plasmodia within the red-blood cell is asexual, it being impossible to detect any constant difference in the appearance of the two conjugants. It occurs between two young amebula and is completed, so far as can be seen, by the permanent union of both nucleus and protoplasm. The process may be observed in all acute initial infections and in recurrent infections, but only in latent infections, in the peripheral blood, just before the appearance of clinical symptoms.

Continued study of this subject has confirmed the belief I have already expressed, that the process is intended to maintain the malarial infection in the blood of man, and that it occurs whenever the races of plasmodia are in danger of dying out from repeated sporulation in the usual manner; it is therefore most frequently observed in the latter part of an acute attack, instead of before the appearance of clinical symptoms. I am also convinced that in this process there

lies, in all probability, the explanation of latent and recurrent malarial infections.

As has been shown, conjugation occurs in many, if not almost all, organisms, when unfavorable conditions arise, such as exhaustion from repeated division, insufficient nutriment, or the presence of conditions in the environment that are unfavorable to growth in the usual manner; it has also been shown that under such conditions a resting or *zygote* stage succeeds conjugation, in which the usual vital activities are wholly or in part suspended until the conditions again become favorable, when the vatal activities are resumed and reproduction occurs as before.

If we consider carefully the phenomena of intracorpuscular conjugation as seen in the malarial plasmodia it is evident that they conform to those observed during conjugation in many of the Protozoa, and that, if there is any value in analogy, the conditions leading to the process and its significance are similar. Considered in this way I believe that intracorpuscular conjugation is most easily explained, and that the theory of the etiology of latency and recurrence which follows is one that is worthy of careful study and one that is well supported by the known significance of conjugation in other protozoan organisms. Intracorpuscular conjugation in malaria occurs after a series of reproductions by spore-formations, during which time the initial potential energy of the race of plasmodia has gradually declined; during this same period the clinical symptoms of malaria have been present, and the environment of the plasmodia rendered unfavorable, perhaps, by the administration of drugs, such as quinine; as a consequence, decreased ability to reproduce by spore formation leads to intracorpuscular conjugation, and the formation of a resistant form of the plasmodium; the process occurs within the red blood corpuscle, because only here can nutriment be obtained for further development.

When conjugation is completed by the permanent union of the protoplasm and nucleus of the two conjugants, growth occurs at the expense of the red cell, until finally the entire cell is destroyed, and the round, pigmented organism is liberated; this form now, in all probability, becomes encysted and enters upon a resting or *zygote* stage, and it is my belief that it is this stage that has been

considered by Schaudinn[49] as a *macrogamete*, which by parthenogenesis gives rise to recurrences.

This stage is probably more resistant to injurious influences, such as quinine, than other forms of the plasmodia, and may continue unharmed in one of the internal organs, as in the spleen, or, more probably, in the bone marrow, for long periods of time. When conditions are favorable the cyst (for it is probable that the organisms *are* encysted) ruptures, and liberates a generation of spores which have developed within it; these young plasmodia penetrate the red blood corpuscles and reproduce, as is usual, by spore formation. Latency is thus rendered possible by the resistance of the resting or *zygote* stage, and recurrences are due to the liberation and subsequent sporulation of the young plasmodia. In cases in which this form of conjugation is present, numerous large pigmented plasmodia are observed, both within and external to the red cells, which show no evidence at any time of segmentation or flagellation; in these the pigment is small in amount and distributed irregularly in very fine granules; they are often very numerous in the blood of cases in which intracorpuscular conjugation is present and are not observed in the blood of other cases; in aestivo-autumnal infections these bodies are only seen in the blood obtained by splenic puncture. These bodies are probably the ones concerned in the production of the latent forms and eventually become encysted and situated in some internal organ or in the bone marrow.

It would seem that the growth of the conjugating form within the red cell is rapid until the destruction of the cell, when the plasmodium is liberated and enters upon the resting or *zygote* stage; the duration of this stage probably varies with conditions, but I believe that it lasts for several days at least.

Reasoning from analogy this stage must continue for some time, for, as has been shown from the Protozoa, by Bütschli, Maupas, and Hertwig, the two conjugating individuals might by simple division give rise to many others during the time occupied in conjugation, and this is true of every organism in which asexual conjugation occurs, so far as I know. It would, indeed, be strange if the malarial plasmodia were exceptions to so general a rule, and therefore it follows that the resting or *zygote* stage must continue for several days, and that

intracorpuscular conjugation, admitting that it produces this form of the plasmodia, is the cause of latency and recurrences. The periods of time between relapses, which vary somewhat, but are remarkably uniform when quinine has not been administered, are explained by the time consumed in the completion of the process of intracorpuscular conjugation and the development of the young plasmodia within the encysted *zygote*, while the marked irregularity in the period between relapses after quinine has been administered, is explained by the liberation of the young plasmodia only when the environment is favorable, i. e., when the quinine has been discontinued, or the quantity administered greatly diminished. We thus see relapses quickly follow the discontinuance of quinine in all malarial regions, and Celli, in his suggestion that relapses may depend upon asexual forms which remain inert in some of the viscera, stated what is in all probability the true explanation of recurrences in malaria, the inert asexual bodies of Celli being the *zygote* or resting form of the plasmodia, produced by intracorpuscular conjugation. That such a latent or resting form of the parasites of malaria is present somewhere in the body in malarial infection is proven by the fact that the withdrawal of quinine in cases which have been taking it for weeks is often followed by a relapse and the reappearance of the parasite in the blood; it is impossible to believe that normal schizogony has occurred in suchcases for weeks, even when quinine has been administered in large doses, without producing symptoms, and while, as I have shown,[50] in discussing the pathology of latent malarial infection, schizogony does occur within the spleen without symptoms being produced, it should be remembered that in the cases in which this was observed no quinine was being administered, and symptoms would probably have soon appeared if the patients had lived. The administration of quinine, even in very moderate doses, has a very marked effect upon the process of intracorpuscular conjugation as observed in the peripheral blood, the conjugating parasites disappearing, perhaps collecting in some internal organ, while in those cases in which the process has not appeared it is never observed if quinine be administered. The large pigmented forms which I believe result from conjugation, show no morphological changes after the administration of quinine.

From my studies of this subject I believe that the following conclusions are justified regarding the significance of intracorpuscular conjugation:

1. Intracorpuscular conjugation is the chief cause of the maintenance of malarial infection.

2. It maintains malarial infection by producing a resting or *zygote* stage of the plasmodia, within the human body, which is resistant to quinine and other injurious influences.

3. It is the cause of latency and recurrences of malarial infection, the *zygote* stage remaining dormant or "latent" until conditions are favorable, when it gives birth to several young plasmodia, thus causing a recurrence of the infection.

These conclusions are justified by the following considerations:

1. The presence of the process in all acute and recurrent infections.

2. The fact that during the time consumed in conjugation in all other Protozoa, provided the conjugation is asexual, many generations of the organism could have been produced by division or sporulation in the usual manner.

3. The fact that such a resting or *zygote* stage must exist, as proven by the recurrence of the infection after the discontinuance of quinine given for long periods of time.

4. The fact that in cases which have been treated at once with sufficient doses of quinine and for a sufficiently long period, intracorpuscular conjugation is never seen, and in such cases relapses are very rare, if they ever occur.

5. The presence of numerous large pigmented bodies in the blood in cases in which the process is most marked, both intra- and extracellular, and which are not seen in cases in which the process is absent.

6. The argument from "analogy" which indicates that the significance of the process of asexual conjugatoin in the malarial plasmodia is similar to the same process in other of the Protozoa.

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EXPLANATION OF PLATE 2.

A. SCHIZOGONY OF MALARIAL PLASMODIUM. ACTIVE CYCLE.

1 to 7.—Active cycle of *schizogony*, showing development of the *merozoite* into *schizont* after penetrating the red-blood corpuscle, and the development and final sporulation of the *schizont*.

B. SCHIZOGONY OF MALARIAL PLASMODIUM. LATENT CYCLE.

1^a to 9^a.—Latent cycle of *schizogony*. Two of the *merozoites* (6) enter a red blood corpuscle and there conjugate (1^a to 6^a), producing a single organism which grows until the red-blood corpuscle is destroyed, when it is liberated and eventually becomes encysted, developing within the cyst several young plasmodia (7^a) which, when conditions are favorable, are liberated by the rupture of the cyst (8^a) and, penetrating the red corpuscles, undergo development as in the active cycle of the *schizogony*.

C. SPOROGONY OF MALARIAL PLASMODIUM.

1^b to 21^b.—Illustrating steps in the *sporogony* of the malarial plasmodium within the mosquito.

1^b to 6^b.—Development of the *macrogamete* and the *microgametocyte* which occurs within man.

7^b and 8^b.—Development of the *macrogamete* and *microgamete* (free flagellum) within the stomach of the mosquito.

9^b.—Fertilization of the *macrogamete* by the *microgamete*.

10^b to 17^b.—Development of the *oökinete*, *oöcyst*, *sporoblast*, and *sporozoites*.

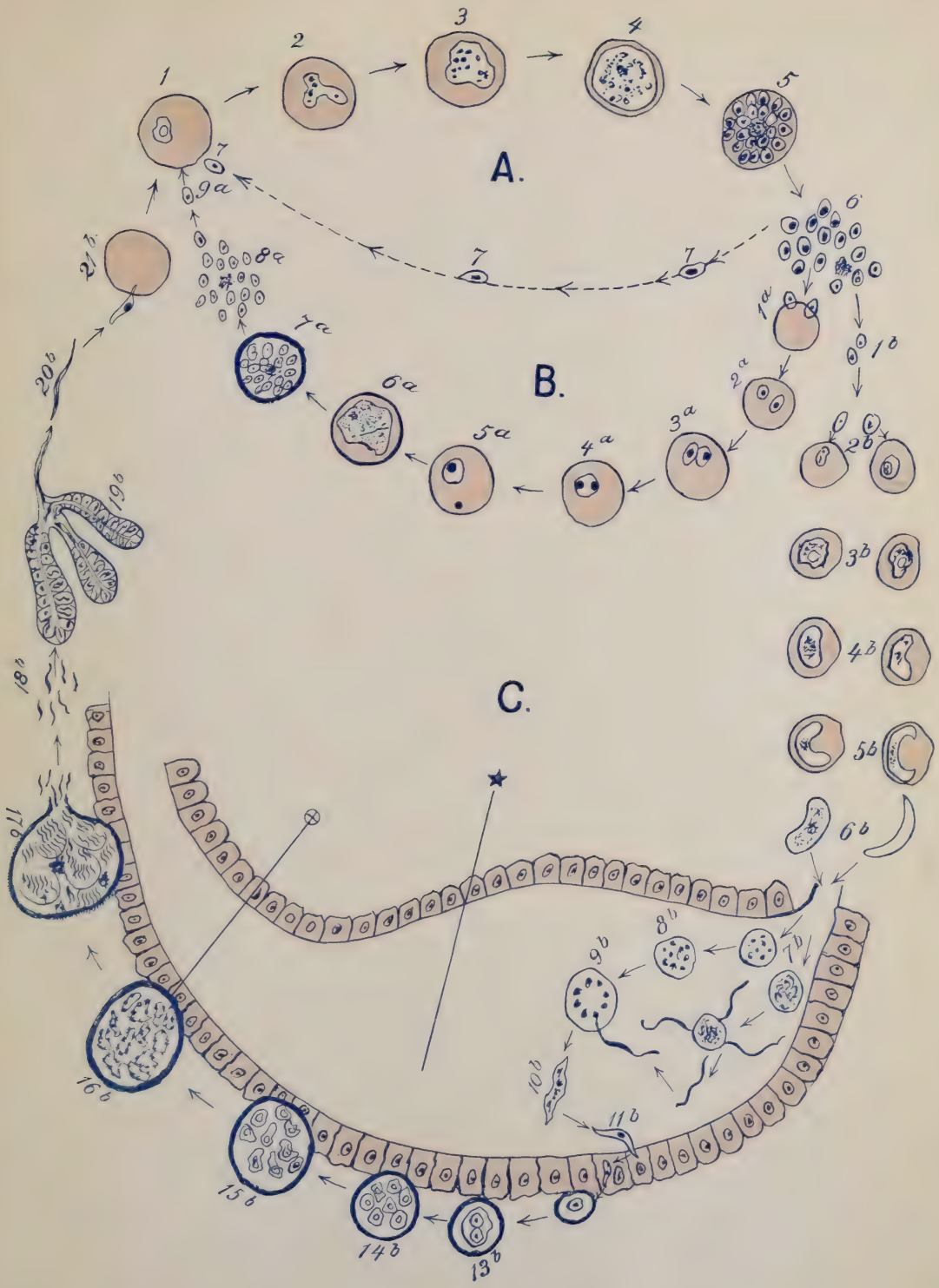
18^b.—Liberated *sporozoites*, which reach the salivary gland of the mosquito (19^a).

20^b and 21^b.—*Sporozoite* passing from salivary gland and eventually penetrating the red blood corpuscle of man.

*—Interior of stomach of mosquito.

†—Wall of stomach of mosquito.

PLATE 2.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OBSERVATIONS ON THE VIRUS AND MEANS OF TRANSMISSION OF ROCKY MOUNTAIN SPOTTED FEVER.*†

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INVESTIGATIONS into the nature of Rocky Mountain Spotted Fever its etiology and method of transmission, have been carried on by the writer since April, 1906.

Previous to these investigations the disease had been studied extensively by Wilson and Chowning,[1] and to some degree also by members of the Public Health and Marine Hospital Service. I shall refer to these investigations only to say that Wilson and Chowning described the disease as a pyroplasmosis, and advanced the important theory that man is infected by the bite of the "wood-tick" which infests the mountainous regions of Montana and adjacent Rocky Mountain States. They furnished no experimental proof of the correctness of the theory. Following the report of Wilson and Chowning, Stiles,[2] of the Public Health and Marine Hospital Service, studied the disease and failed utterly to find the pyroplasma of the former investigators. Stiles discredited also the theory of transmission by the tick, but without experimental evidence to refute the theory.

This was the status in relation to the etiology and means of transmission of the disease when my studies were undertaken. The results which I have obtained have been described briefly in three communications to the *Journal of the American Medical Association.*[3]

The essential points presented in these articles are the following:

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[1] *Jour. Amer. Med. Assoc.*, 1902, 39, p. 131; also *Jour. Infect. Dis.*, 1904, 1, p. 31.

[2] *Pub. Health and Mar. Hosp. Bull.*, No. 20, 1905.

[3] (1) "The Study of 'Rocky Mountain Spotted Fever' (Tick Fever) by means of Animal Inoculations," *Jour. Am. Med. Assoc.*, July 7, 1906, 47, pp. 33–36; (2) "The Transmission of Rocky Mountain Spotted Fever by the Bite of the Wood-Tick (*Dermacentor occidentalis*)," *ibid.*, August 4, 1906, 47, p. 358; (3) "Further Observations on Rocky Mountain Spotted Fever and *Dermacentor occidentalis*," *ibid.*, October 6, 1906, 47, pp. 1067–69.

The disease was transmitted to guinea-pigs from three different cases, and to monkeys from two different cases by inoculation with defibrinated blood from the patients (1, 3). From the last of the three cases the disease has been maintained in the laboratory by alternate passage through the monkey and the guinea-pig, by means of inoculations with blood or emulsions of organs (3). The first attempts to maintain the infection by direct inoculation from guinea-pig to guinea-pig failed, possibly for reasons which will be referred to below. Rabbits were found not to be susceptible to an appreciable degree, and the same was true of white mice and white rats. In a preliminary experiment the virus did not pass through a Berkefeld filter, although the unfiltered serum was infectious. The anatomical similarity of the disease produced in the guinea-pig and monkey to the conditions observed in man, and the incubation period and course of fever induced by inoculation, were considered sufficient evidence of the genuine transmission of the disease to these animals (1, 3). In a single experiment a tick, female, was found to be the carrier of the disease from one guinea-pig to another. This result was considered as tentative (2).[1]

The virus was found to be distributed in all of the visceral organs, the most vascular organs (liver, spleen, and bone marrow) and the kidney apparently being the most infectious (3).

It was found that an active immunity which possibly is relative in degree, is established in the monkey and guinea-pig by one attack (3). The life-history of *Dermacentor occidentalis* was followed under laboratory conditions. This tick passes through a larval stage and moults twice subsequently, before reaching the adult form, the cycle from egg to adult requiring about three months under the modified conditions, which prevailed (3). The tick left the host in order to moult.

It is the purpose of this paper to present further observations which have been made in relation to the distribution of the virus in the body fluids, its viability and resistance under different conditions, its filterability and certain other properties, the possibility of transmission by means of the bite of the male tick, and in addition, the preservation of the virus by uninterrupted passage through the guinea-pig.

[1] King also has reported transmission by means of the female tick (*Pub. Health Reports*, ...).

THE DISTRIBUTION OF THE VIRUS IN THE BLOOD.

Rocky Mountain Spotted Fever is unquestionably a systemic infection, since the disease may be transmitted by the inoculation with blood and with emulsions of the solid organs.

A study of the distribution of the virus in the blood was undertaken with the hope that the question of an exclusive or predominant erythrocytic infection, such as a pyroplasmosis, might be affirmatively or negatively determined. Although the experiments reported are not quantitative in character, it is thought that the sum total of results renders the pyroplasma theory open to suspicion. Quantitative work is very difficult from the fact that the concentration of the virus in different animals is subject to considerable variation; this may be as great as a tenfold variation, the minimum fatal dose of defibrinated blood in one instance being 0.05 c.c. and in another 0.5 c.c.

The following results have been obtained in regard to this phase of the work:

1. It is impossible to free the blood cells of defibrinated blood from the virus by 10 to 12 washings with physiologic salt solution (Table 3). Repeated washing, however, seems to decrease the virulence of the corpuscles of infected blood (Table 7).

3. The serum obtained from defibrinated blood or from spontaneous clotting is infectious in doses of 0.5 c.c. or even less (Table 5).

4. Prolonged centrifugation of the serum (6 hours) does not free the overlying portion from the virus (Table 8).

5. An exudate of leucocytes in the pleural or peritoneal cavity of the infected monkey or guinea-pig, caused by the injection of aleuronat or bouillon, is infectious for the guinea-pig. The sediment of such an exudate is not deprived of its infectiousness by repeated washings, and the overlying fluid remains infectious in spite of prolonged centrifugation (Table 6).

2. As washing advances a point is reached at which the virus is not separated from the corpuscles in infective quantities by shaking with salt solution. An experiment showed that a fluid of the 10th washing was not infectious in quantities of 5 and 10 c.c., whereas the cellular sediment caused the disease in doses of 0.7 and 1.5 c.c. (Tables 3 and 4).

The following selected experiments are given as illustrative of the preceding statements:

TABLE 1.

CONTROL EXPERIMENT. INOCULATIONS WITH FRESH UNDEFIBRINATED BLOOD OF MONKEY XIV.

Guinea-Pig No.	Dose	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
150	0.05 c.c.	3–4 days	12 days	Death	Typical*
151	0.1	3–4	10½ "	"	"
152	0.3	3–4	11 "	"	"
153	0.7	3–4	9 "	"	"

* The following conditions at autopsy are recognized as typical for spotted fever in the guinea-pig: Generalized enlargement of the lymph glands including those of the mesentery and mediastinum. Either extreme congestion of, or hemorrhage into, the lymph glands. Enlargement of the spleen, which may reach the size of two or four times the mass of the normal spleen. The spleen is much congested and cyanotic, fairly homogeneous and of moderate consistency, i. e., neither soft nor hard. The kidneys suprarenal glands, and liver are somewhat enlarged and congested. The right heart and veins are heavily engorged. The lungs show no changes. The meninges are moderately reddened. The bone marrow is rich in rather dark red blood. Very frequent, but not constant changes are hemorrhages into the skin of the external genitalia, and, in males, into the testicles and their coverings. Hemorrhage also occurs with some frequency into the suprarenal gland, and less often, into the liver. If visible colonies appear on a plate of the heart's blood which is made soon after death, or if peritonitis or other severe local inflammation is found, the animal has been disregarded.

TABLE 2.

CONTROL EXPERIMENT. INOCULATIONS WITH FRESH DEFIBRINATED BLOOD OF MONKEY XIV.

Guinea-Pig No.	Dose	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
154.....	0.05 c.c.	5–6 days	13 days	Death	Typical
155.....	0.1	5–6	11 "	"	"
156.....	0.3	3–4	12 "	"	"
157.....	0.7	3–4	11 "	"	"

TABLE 3.

INOCULATIONS WITH WASHED DEFIBRINATED BLOOD OF MONKEY XIV: TEN WASHINGS, THE ORIGINAL VOLUME BEING RESTORED.

Guinea-Pig No.	Dose	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
162†.....	0.3 c.c.			Not sick	
163.....	0.7	5 days	8 days	Killed	Typical
164.....	1.0			Not sick	
165.....	1.5	5–6	13	Death	"

†Not sick. Died later, following rectal prolapse.

TABLE 4.

INOCULATIONS WITH OVERLYING SALT SOLUTION OF THE TENTH WASHING OF BLOOD USED IN TABLE 3.

No.	Dose	Result
167.....	10 c.c.	Not sick

TABLE 5.

INOCULATIONS WITH SERUM FROM BLOOD OF MONKEY XIV, THE SERUM BEING OBTAINED BY DEFIBRINATION AND CENTRIFUGATION FOR ONE HOUR.

Guinea-Pig No.	Dose	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
158	1.0	3–4 days	11 days	Death	Typical
159	2.0	3–4	13	"	"
160†	3.0				
161	4.0	3–4	14	"	"

† Accidental death in 4 days.

TABLE 6.

INFECTIOUS PROPERTIES OF LEUCOCYTIC EXUDATE FROM MONKEY XIV, CAUSED BY AN INJECTION OF ALEURONAT.

Guinea-Pig No.	Dose and Material Inoculated	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
147.....	2 c.c. of unaltered exudate	6 days	13 days	Death	Typical
148.....	5 c.c. of leucocyte suspension, washed 3 times	6	14	"	"
149.....	4½ c.c. of fluid from first centrifugation	6–7	21	Recovery	

TABLE 7.

THE EFFECT OF REPEATED WASHING OF BLOOD IN DECREASING ITS VIRULENCE. MIXED BLOODS OF MONKEYS VII AND VIII 12 C. C. INJECTED INTO EACH GUINEA-PIG.

Guinea-Pig No.	Washings	Incubation Period	Duration from Date of Inoculation	Result	Autopsy
44.....	1	2 days	7 days	Death	Typical
45.....	2	4	10	"	"
47.....	5	5	14	"	"
46.....	10	5	16	"	"

TABLE 8.

ATTEMPT TO REMOVE INFECTIOUS PROPERTIES OF SERUM BY CENTRIFUGATION FOR SIX HOURS AT THE RATE OF 2,000 REVOLUTIONS PER MINUTE. 5 C.C. OF SERUM FROM MONKEYS VII AND VIII

Guinea-Pig No.	Injection	Incubation Period	Duration	Result
50.....	Centrifugation serum	7–8 days	20 days	Recovery
51.....	Final 0.5 c.c. of above	5	16	"

The tenacity with which the virus associates itself with the blood corpuscles suggests that a certain proportion of the parasites may be within either the leucocytes or the erythrocytes. This suggestion seems the stronger since, as shown in Guinea-pig 167 (Table 4), a certain proportion of the virus is not readily separated from the corpuscles by moderate agitation with salt solution, but remains

rather firmly associated with the blood cells (Table 3). In relation to this fact, however, the following points are to be observed: First, the density and viscosity of serum is considerably greater than that of 0.85 per cent salt solution, and although the virus cannot be separated from serum readily by centrifugation, such separation could be more readily accomplished in salt solution. Hence it is still possible that the absence of the virus in pathogenic quantity from the salt solution of the 10th washing may be caused in large part by the sedimentation necessary for the separation of the corpuscles. Not only must the difference in the density of serum and salt solution cause a wide difference in the ease with which microbes may be sedimented from their solutions, but the coarse physical structure of serum must interfere greatly with the sedimentation of minute particles. In the second place it is readily conceivable that minute organisms may adhere to the external surface of erythrocytes and leucocytes so firmly that moderate agitation in salt solution does not cause their separation in pathogenic quantities.

In an experiment, the protocol of which is not given, 0.5 c.c. of serum obtained after spontaneous clotting caused fatal infection in the guinea-pig. This would suggest a general plasmatic infection rather than one which is essentially cellular.

Since a leucocytic exudate practically devoid of erythrocytes is infectious, it seems probable that the condition could not well be considered as an essential involvement of the erythrocytes. Furthermore, from the fact that the virus exists in the fluid portion of the leucocytic exudate in pathogenic quantities, the infection could hardly be considered as essentially leucocytic in character.

Although it is desirable to wait until certain quantitative experiments are completed before drawing positive conclusions as to the situation of the virus, I believe the qualitative results described above indicate a general plasmatic infection rather than an essential invasion of blood cells.

FILTRATION AND INTOXICATION.

Filtration experiments have been continued with variations in conditions. Small Berkefeld candles have been used exclusively and for the most part those which were fresh from the factory. In case a

filter had been previously used, it was washed with distilled water in both directions, subjected to prolonged boiling in a solution of sodium carbonate, and washed first with salt solution and then with distilled water in both directions, before being used again. It has seemed useless to employ porcelain filters so long as the virus has not been passed through the more porous Berkefeld filters.

No greater pressure has been used than that obtained from a vacuum water pump, with a pressure of from 30 to 40 pounds in the mains. In all experiments except one, the filters have been kept covered with the serum by drawing the latter into a pipette, then letting it fall over the surface of the filter. In the exception mentioned, the filter was covered with a rubber tube which extended one-half to three-quarters of an inch above the height of the filter, the serum being fed into the cup made by the projection of the tube. In this particular instance the attempt was made to filter the serum undiluted. After a time, however, the filtration proceeded so slowly that salt solution was added. In the remaining experiments an equal quantity of salt solution was added to the serum before filtration was begun, and in all cases the filter has been washed out with several cubic centimeters of salt solution after the serum had passed through. The serum of infected monkeys has been used throughout for filtration experiments, the serum being obtained by defibrination and centrifugation. In all instances the infectiousness of the unfiltered serum has been determined by control experiments and the serum has been used as soon as possible after obtaining it.

In no instance has infected serum even in quantities of 6 to 12 c.c. been infectious for the guinea-pig after being filtered in the manner described, although 0.5 c.c. of fresh defibrinated blood have not failed to cause the disease (see Table 9, as an illustration).

TABLE 9.

FILTRATION EXPERIMENT. SERUM OF MONKEY XII. 5 C.C. OF THE DILUTED SERUM WERE INJECTED INTRAPERITONEALLY AND THE REMAINING PORTION SUBCUTANEOUSLY.

Guinea-Pig No.	Volume of Serum	Volume after Dilution	Incubation Period	Result	Autopsy
Filtered....	7 c.c.	9—10 c.c.		Not sick	
Unfiltered..	7	9—10	7 days	Death	Typical

In view of the possibility that minute forms of the organism, capable of passing through the Berkefeld filter, might exist within the

erythrocytes or leucocytes, whereas only larger forms might be extracellular, an attempt was made to free such hypothetical minute forms by crushing the blood cells in a porcelain ball-mill. Inasmuch as the result of this experiment points to the existence of a toxic substance in infected blood, the details may be given.

Defibrinated blood from monkey No. XIV was washed 10 times with sterile physiologic salt solution in order to get rid of the serum. This washed blood proved to be infectious for guinea-pigs in doses of 0.7 and 1.5 c.c. (see Table 3).

The washed cells from 25.2 c.c. of blood were ground in the ball-mill for six hours. The mass was removed from the mill by fractional washing with salt solution, centrifugated to get rid of porcelain sand, the latter then being washed fractionally, and the total volume of fluid being made up to 50.4 c.c. by additional salt solution. The fluid was dark red in color, cloudy, and no cells could be recognized by microscopic examination. Some of this fluid, representing 50 per cent of blood in volume, was injected into guinea-pigs in doses of 1.4 2.0, 5.0, and 10.0 c.c., the effects of which are shown in Table 10.

TABLE 10.

TEST OF THE INFECTIOUSNESS OF THE WASHED AND GROUND-UP CORPUSCLES OF MONKEY XIV.

Guinea-Pig No.	Volume Injected	Equivalent in Normal Blood Volume Less Estimated 10% Loss	Result
170.....	1.4 c.c.	0.63 c.c.	Slight febrile reaction beginning on the second day after inoculation. Recovery
171.....	2.0	0.9	Moderate febrile reaction beginning the first day after inoculation. Recovery
172.....	5.0	2.25	Moderate febrile reaction beginning the first day after inoculation. The primary temperature subsided, but in 6 days a course of fever developed which persisted for 9 days. Recovery
173.....	10.0	4.5	Similar to that of 172, but died on the 27th day after inoculation. Death due to extraneous infection, as shown by culture

The remaining portion was passed through a fresh Berkefeld filter about three hours being occupied in filtration. Filtration proceeded rapidly at first, but more slowly later, as the fluid became more concentrated in insoluble albuminous particles. The filtrate was dark red and perfectly clear. The entire amount was injected intraperitoneally into two guinea-pigs at interrupted periods. (Table 11.)

TABLE 11.

SHOWING THE TOXIC EFFECTS OF THE FILTRATE OF WASHED AND GROUND-UP CORPUSCLES FROM THE BLOOD OF MONKEY XIV.

Guinea-Pig No.	Volume Injected Fractionally	Equivalent in Normal Blood Volume Less Estimated 15% Loss	Result
174.....	15 c.c.	6.4 c.c.	Death in 4 days following febrile reaction. Blood sterile
175.....	20	8.5	Death in 6 days following febrile reaction. Blood sterile

In comparing Tables 3, 10, and 11, which represent experiments performed with the same blood, one gains the impression, first, that grinding the blood in the mill had almost entirely destroyed its infectiousness, and second, that with the destruction of the infectiousness of the blood the latter manifested a rather pronounced toxicity.

In support of the first point, it may be noted in Table 3 that the blood before it was ground up produced typical infections in doses of 0.7 c.c. and 1.5 c.c., whereas after it was ground up, its infectiousness had been largely destroyed, as seen in Table 10. This effect, possibly, is due to an actual crushing of the organism in the mill.

In support of the second point, we have to note first the primary fever which developed in the guinea-pigs of Table 10, and second, the toxicity of the filtrate as shown in Table 11. In order to determine whether the intoxication seen in the animals of Table 11 was due to microbic poison or to the action of the proteids of monkey corpuscles on the guinea-pig, 12 c.c. of normal monkey blood were ground up and a filtrate prepared analogous to that injected into guinea-pigs Nos. 174 and 175. The total filtrate, 20 c.c., was injected into a single guinea-pig which had the weight of the former animals. A slight rise of temperature which occurred on the fifth day after injection lasted two days; otherwise there was no disturbance. It may accordingly be concluded that the intoxication of animals Nos. 174 and 175 was not due to the normal proteids of the corpuscles of monkey's blood. One could scarcely consider the condition an infection since the same blood before filtration had shown practically no infectious properties (Table 10). It could hardly be due to soluble toxins in the monkey's blood, since such toxins probably would have been removed by the washings to which the blood had been subjected.

Although further experiments are indicated before positive conclusions are drawn, it seems probable that the intoxication of the animals was due to the liberation of poisons by the crushing of the virus in the ball-mill.

RESISTANCE TO HEAT.

Heating experiments have been performed at temperatures of 45° and 50° C. It was necessary to use rather large quantities of blood in order to be certain that a fatal quantity of organisms was being dealt with. On this account it was preferable to use relatively low temperatures over rather long exposures in order to insure as complete diffusion of the heat as possible.

Results.—In an experiment in which 3.5 c.c. of blood were heated at 45° C. for 5, 10, 15, 20, 25, and 30 minutes, all animals died of spotted fever. In two experiments in which the blood was heated at 50° C., the infectiousness of the virus was destroyed in 25 minutes in one series and in 30 minutes in another.

RESISTANCE TO DESICCATION.

The following is the technique used in desiccation experiments: Uniform quantities of blood are distributed into open Petrie dishes which are placed in a desiccator over sulphuric acid and dried as quickly as possible under vacuum at room temperature. Desiccation requires from 18 to 24 hours, depending on the degree of exhaustion of the bell-jar. When desiccation is complete the plates are placed in an ordinary sulphuric-acid desiccator in the ice-chest.

Results.—Two series of experiments showed the loss of pathogenicity at some time between 24 and 48 hours after complete desiccation.

VIABILITY IN THE ICE-CHEST.

The M. L. D. of the blood of Monkey No. XV, which when drawn was about 0.1 c.c., had increased noticeably in five days, reached 2.0 c.c. in 11 days, and in 15 days 3.0 c.c. failed to produce infection. The blood was kept in the ice compartment of the ice-chest. In another instance 5.0 c.c. retained infectiousness for 16 days.

TRANSMISSION BY MEANS OF THE MALE TICK

The possibility of transmission by means of the male tick (*Dermacentor occidentalis*) has been demonstrated conclusively in a recent experiment. The tick was one which had been raised from the egg in the laboratory, the life-history of the brood having been published previously (*loc. cit.*).

Infection of the tick was accomplished by feeding on two sick guinea-pigs in the following way: On October 16 it was placed on the ear of guinea-pig No. 107, where it remained for about 12 hours at the end of which time the guinea-pig died. Two days later it was placed on the ear of guinea-pig No. 121, where it remained for about 20 hours, or until the guinea-pig died. After an intermission of three days the tick was placed on the ear of a healthy guinea-pig (No. 169), and the latter died in 13 days showing changes which have been recognized as characteristic of spotted fever. However, since an adventitious epidemic had developed among the guinea-pigs and since areas of focal necrosis found in the spleen of guinea-pig No. 169 were not entirely typical for spotted fever in the guinea-pig, the experiment was not considered conclusive and the animal was discarded. On November 7, 17 days after the tick had been removed from the infected guinea-pig (No. 121) it was again placed on a healthy guinea-pig (No. 182). It was allowed to remain attached for $3\frac{1}{3}$ days, after which it was removed. Ten days after the tick was placed on the guinea-pig the latter suddenly developed high fever and died in five days, showing those anatomical changes which have proved to be diagnostic of experimental spotted fever.

Autopsy.—Scrotum moderately hemorrhagic and very cyanotic. Hemorrhagic condition is seen best by the naked eye in cutting through the skin. Testicular coverings are moderately congested and the anterior pole of the testicles deeply infiltrated with blood. The axillary, inguinal and mesenteric lymph glands are enlarged and hemorrhagic. The spleen is several times the mass of the normal spleen and cyanotic in color. The kidneys are congested and cyanotic; suprarenal glands are enlarged; liver enlarged, congested and cyanotic. The lungs and heart show no appreciable changes. The meninges are slightly reddened. At the point of the tick bite is a necrotic crusted wound about one-fourth inch in diameter. Cultures from the heart and peritoneum yielded no growth.

From the organs of this guinea-pig inoculations were made into two other guinea-pigs, one of which died in seven and the other in

eight days, both showing typical anatomical and clinical phenomena of spotted fever. Monkey No. 17 which was also inoculated from guinea-pig No. 182 ran a typical course and presented extensive scrotal hemorrhage.

From the second generation in guinea-pigs and also from the monkey, inoculations were made into a third generation, the members of which ran typical courses. This is being continued by successive inoculations of the guinea-pig.

The experiment is regarded as conclusive.

CONTINUOUS PASSAGE THROUGH THE GUINEA-PIG.

In a previous article (*loc. cit.*) my failure to keep spotted fever alive by the successive inoculation of guinea-pigs was referred to. In the earlier attempts fresh inoculations were made only as the guinea-pigs were about to die or after they had died. The possibility was recognized that the quantity of living virus in an infected animal may be greater early in the course of the disease than at the time of death, hence at a convenient time the attempt was made to perpetuate the infection in the guinea-pig alone by inoculation with blood or organs taken on the third to the fifth day after fever had begun. This method has proved entirely successful through five and into six generations of guinea-pigs. Hence it seems probable that the monkey can be dispensed with for the purpose of maintaining the disease in the laboratory.

SUMMARY.

Rocky Mountain spotted fever is transmissible to the guinea-pig and monkey by the inoculation of defibrinated blood of patients suffering from the disease.

The virus may be kept alive in the laboratory either by alternate inoculation of monkey and guinea-pig, or by continuous passage through the guinea-pig by observing the method described above.

The disease is transmissible from one animal to another by means of the bite of either the male or female tick (*Dermacentor occidentalis*).

One attack of the disease establishes a rather high degree of immunity to subsequent inoculation.

Attempts to pass the virus through Berkefeld filters have failed.

The parasites are not located essentially in either erythrocytes or leucocytes but are present in the body fluids generally.

By grinding infected blood in the ball-mill infectiousness is largely destroyed; in this process there is some reason to think that the organisms are crushed and that toxic substances are thereby liberated.

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A SPIROCHETE IN PRIMARY AND TRANSPLANTED CARCINOMA OF THE BREAST IN MICE.*

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IN the early spring of 1905 the writer first observed in the epithelium of a retrogading transplanted tumor of the Jensen series, certain curious structures which were the subject of a publication by G. N. Calkins and G. H. A. Clowes under the title "Some Artefacts in Mouse Carcinoma."[1] The material in which the structures in question were first seen was hardened in an excess of mercury and contained, besides the structures about to be specified, curious deposits in crystalline form, both intra- and extra-cellular. The structures which attracted our attention at that time were very fine filaments of varying length, frequently possessed of clubbed ends, located in the protoplasm. They are illustrated by Figs. 5 and 6 of Plate 17, and by drawings by Calkins in Figs. 6, 7, 8, 9, and 10 of Plate 18.

The first impression which we had of these structures was that they must be some sort of parasitic form as yet unrecognized, but our inability to distinguish them from the deposits of mercury in the surrounding tissues, and in the nuclei and protoplasm of the cells of the

* Received for publication, March 12, 1907.

[1] *Jour. Infect. Dis.*, 1905, 2, p. 555.

tumor, led to the conclusion by Calkins and Clowes that they were artefacts, and the specimen was thereupon described and published.

So deeply impressed was the writer that certain of these forms must be parasitic that he never abandoned that point of view; and that Clowes and Calkins were also of the opinion that the bodies represented unusual structures impregnated with mercury is shown by the following sentence in their publication, on p. 559: "While these deposits are to be interpreted as artefacts, the fact must not be overlooked that something of an unusual nature is present in these cancer cells and tissues, upon which the salts of the fixing agent work in forming the deposits of various kinds."

Being deeply impressed with the possible significance of these curious filamentous structures in the vacuoles of the cells of the transplanted mouse tumor, and having made various attempts to find a method which would better demonstrate them if present, the writer at once saw, on the publication of Levaditi's article,[1] a close resemblance between these filamentous structures and the involution forms of *Spirochaeta pallida* so frequently encountered in syphilitic tissue. Thereupon a systematic investigation of all the transplanted mouse tumors in the State Cancer Laboratory was undertaken by the Levaditi method. The results of this systematic examination are here reported.*

An examination of transplanted tumors from three separate strains which are at present in existence in this laboratory has shown the constant occurrence of a characteristic organism in the transplanted tumors of each. The tumors from which these strains are derived are first the famous Jensen, which is now, in our hands, in its twentieth generation; a very virulent tumor known as the Brooklyn tumor, in its twentieth generation; and a less virulent tumor, known as the Springfield tumor, in its twelfth generation. All three of these tumors are carcinomata of the breast. They present practically identical characteristics, being carcinoma solidum, with occasionally adenomatous types.

[1] *Ann. de l'Inst. Pasteur*, 1906, 20, p. 41.

*The title of this paper was on the program, but it was not read, at the meeting of the British Medical Association in Toronto, August 22, 1906. The facts in substance were presented at a meeting of the Academy of Medicine in Cleveland, January 11, 1907, and before the State Medical Society of New York at Albany, January 29, 1907.

The tumor known as 3^{10}, in which the original threadlike structures impregnated with mercury were discovered in March, 1905, belongs in the second generation of the Jensen strain. We have examined by the Levaditi method 914 E, 914 G, 915 A, and 915 D, out of the fourteenth generation of this tumor. All of these transplanted tumors were movable beneath the skin and uncontaminated. At the margin of the tumors in the Levaditi sections is found in the immediate neighborhood of the infiltrating edge of epithelium, a small spiral organism from 2.5 to 7.8 μ in length, with from four to as high as 13 or 16 nodes or corkscrews. The organism is 0.6 μ in average width; the ends are slightly rounded, and there is no evidence of an undulating membrane or flagellum.

Of the Springfield tumor 900 F, 921 F, 921 H, from the sixth generation, were subjected to the Levaditi method. An organism was found at the margin which was identical in appearance with that found in the Jensen tumors, bore the same relation to the epithelium, and was distributed in the same manner.

Of the Brooklyn tumor 741 A from the ninth generation, 763 E eleventh generation, 918 G thirteenth generation, 904 K thirteenth generation, 935 H fourteenth generation, 918 C fourteenth generation, 1039 E fifteenth generation, 1088 C sixteenth generation, 1113 A seventeenth generation, were examined by the Levaditi method. In every section of every one of these tumors the same characteristic spirochetes were found as described in the Jensen and Springfield tumors. These were in great numbers about the margin of the tumor in the connective tissue immediately adjacent to the epithelium. The organism is also occasionally found in the stroma or between the epithelial cells through the substance of the tumor, but the characteristic distribution is in the zone of round-celled infiltration and between the epithelial cells at the infiltrating edge of the tumor. Some of the larger of these tumors showed in their centers the usual areas of necrosis. A careful examination of these areas of necrosis failed to show the presence of any microorganisms of any kind.

Having determined the practically constant occurrence of this organism by the Levaditi method, attempts were made to stain it by Giemsa, by the various flagella stains, or by Wright's method, all

without success, although the material which was used was known to contain large numbers of organisms. The organism could, however, be easily detected in fresh material. It is rather difficult to see because of its low refractive index and the high refractive index of the fluid portions of the tumor. Patient search has, however, in every instance in which fresh material has been examined, demonstrated well-defined, actively motile organisms. Measurements made of these fresh, living organisms correspond with those made of the hardened organism in the Levaditi sections. Dr. F. G. Novy, who examined for me a fresh preparation from an uncontaminated mouse tumor, made the following measurements: From 2.5 to 7.8 μ in length and from 4 to 13 turns.

The proper position of this organism and a more definite description of its characteristics have been kindly undertaken by Professor Calkins.

Having determined the constant occurrence of a living spirochete in all of our transplanted mouse tumors, it was of the greatest importance to discover with what frequency they could be found in unattached, uncontaminated primary mouse carcinomata. We have up to the present time examined 10 primary mouse carcinomata. One of these was badly impregnated, so that it was impossible to determine the structure of the tumor satisfactorily, and it was therefore excluded. Of the remaining nine, the organisms have been found in all. They were found with no difficulty whatsoever in five. These are known as G-7, G-E, G-A, G-C, Springfield No. 8, and Buffalo I. In the tumors known as G-10 and G-8 the impregnation was imperfect and the organisms were of a pale brown, but could, however, be definitely distinguished. Tumor G-D was large and on its superior aspect slightly attached to the skin. The greater part of the central portion of the tumor was necrotic; only a very narrow margin at the periphery of the deeper portions of the tumor was in a state of active growth, and in these regions the organisms, although few in number, could be easily found.

It is to be noted that all of these tumors, with the exception of G-D and Buffalo I, were freely movable beneath the skin and small. All the G tumors were obtained at different times from a dealer in Massachusetts, whose animals are frequently affected with carcinoma of

the breast. We have received from this dealer, in the course of the last eight months, 31 tumors. The tumor designated Springfield 8 was a small primary tumor obtained from a dealer in Springfield, Ohio, who has furnished us with three tumors in the last year and a half. It is from this dealer that we have obtained a cage from which in the course of three years have been taken over sixty mice with carcinoma of the breast. The cage with its contents was brought to Buffalo, and since its arrival at this laboratory five mice with cancer of the breast have been removed from it. Both of these series of tumor mice have been previously reported.[1] The Buffalo I tumor was found in a cage of pet mice in the home of a Buffalo physician. To these must be added a primary cancer of the breast found by Calkins in a mouse in his laboratory at Columbia University, New York, which contains the organism.

For control of the work up to this point we have impregnated and sectioned pieces from all the organs and subcutaneous tissues of five supposedly normal mice. A prolonged search of all these tissues has given negative results, but more extensive controls will be necessary before the distribution of this organism, which obviously must be very wide, can be defined.

Before passing to a study of the relation of these organisms to the structure of the tumors in which they occur, it may be well to review the scanty literature on the presence of spirochetes in mouse and other carcinomata.

The first reference to the presence of spirochetes in malignant tumors is found in the report of Hoffman.[2] Under Hoffman's direction Mulzer found spirochetes in the scrapings from a case of carcinoma of the cervix and in two squamous epitheliomata, one from the face and the other from the abdominal aspect. All of these tumors were *advanced and ulcerated.* In all three cases the spirochetes showed coarse gyrations and stained more deeply than *Sp. pallida.* Certain individuals were encountered, however, which did not stain so deeply, and in the size and number of their gyrations closely approximated the latter organism.

Löwenthal[3] also describes the detection of spirochetes by the Giemsa method, on the surface of ulcerated tumors. He calls attention to the fact that they are not only present in human tumors under such conditions, but that he has found them in numbers associated with the usual bacteria on the ulcerated surface of a tumor in the neck of a dog. He calls attention to the similarity of the organism he observed to a small spirochete found in feces. This organism usually stains a pale blue with

[1] *Jour. Amer. Med. Assoc.*, 1907, 48, p. 15.

[2] *Berl. klin. Wchnschr.*, 1905, 42, p. 880.

[3] *Ibid.*, 1906, 43, p. 283.

Giemsa, and stains also with a borax-methylene blue solution which does not stain *Sp. pallida*. The organisms are from 2.5 to 6 μ in length. He could not determine their thickness, but estimated it to be from 0.25 to 0.5 μ. They appear plumper when stained with Löffler's flagella stain. The gyrations are very close together and abrupt. The organism has usually from 4 to 12 corkscrews, and he estimates these to be about 0.5 μ apart. He proposes as the name for this organism "*Spirochaeta microgyrata.*" Up to the present he has been unable to determine the presence of flagella, and an undulating membrane was not visible, but he inferred from the increased plumpness of the organism, when stained with Löffler's flagella stain, that one was probably present. The long examples of the organism were frequently found to consist of two individuals attached at the ends. He considers that it may be readily differentiated from *Sp. pallida*. Besides this organism he frequently found a larger form from 5 to 11 μ in length with corkscrews from 1.5 to 2 μ apart which stains blue with Giemsa. In all cases where Löwenthal detected the spirochetes on the ulcerated surface of tumors he found regularly rodlike, straight or slightly bent, sausage-like structures which in their appearance and form closely resembled the so-called fusiform bacillus which accompanies the spirochetes, known as *Spirillum sputigenum*, so frequently found in the buccal cavity. Several authors have already advanced the view that there is a genetic relation between these fusiform structures and the spirochetes with which they are associated. Löwenthal advocates the same view in connection with the fusiform structures accompanying the small spirochetes found on the surface of ulcerating tumors, for the reason that they invariably accompany these organisms. He states that wherever he found fusiform structures in a smear, on further search he never failed to find the small spirochetes in question.

Borrel[1] found helminthia in two inclosed mouse tumors surrounded by large numbers of leucocytes and endothelial phagocytes. The worms were transported to the tumors through the blood vessels after penetrating the intestinal wall. In the neighborhood of these worms Borrel states that large numbers of spirochetes were present, and in a very cachectic mouse sent from Ehrlich's laboratory, in which the tumor was not ulcerated, he found large numbers of spirochetes. These had coarse gyrations. In the two cases described in Paris the spirochetes were of different form, very small, and with closely packed spirals. The tumors thus examined were movable beneath the skin, were not ulcerated, and at the time of publication were still living. He concluded that it was impossible to draw etiological conclusions from these observations, but he found it of great interest that spirochetes and worms were found in these particular strains.

Spirochetes in unulcerated human cancer, demonstrated by the silver method, have as yet been reported by but one observer, Friedenthal.[2] Friedenthal is of the opinion that the structures, which are clearly spirochetes from his illustrations are not organisms, but represent condensations of the protoplasm impregnated with silver. He obviously published his observations to show that so-called spirochetes of syphilis which Schultze and others hold to be nerve fibers and elastic fibers impregnated by the silver method, are not necessarily all attributable to the misinterpretation. Inasmuch as the structures he described were within the epithelial cells of the tumor, Friedenthal held that some of them were condensations of the protoplasm, etc., and not nerve endings or elastic fibers.

[1] *Comp. rend. de la Soc. de Biol.*, 1905, 58, p. 770.

[2] *Berl. klin. Wchnschr.*, 1906, 43, p. 283.

It will be seen that of these observers Mulzer describes a coarse spirochete on the ulcerated surface of human tumors; Löwenthal describes a coarse and a smaller organism, known as *Sp. microgyrata*, on the surface of ulcerated human tumor and in a tumor in the neck of a dog. None of these authors attributes any significance to the presence of these organisms, as they were found under conditions which could not justify any suggestion that they were other than accidental in their occurrence. Borrel found in two cases a small spirochete in connection with helminthia and an organism with coarse gyrations in a cachectic mouse tumor sent from Ehrlich's laboratory. Borrel does not state whether these tumors were primary or transplanted tumors, but the inference is that they were transplanted. He believed that the organisms were conveyed to the tumors by the helminthia in the first two cases, and drew no conclusions as to their possible etiological significance in any of the three cases described.

Besides spirochetes associated with mouse tumors, Wenyon[1] describes a spirochete which he found in the blood of a gray mouse in the Pasteur Institute. From this mouse he succeeded in infecting other mice; in these both the blood and the spleen contained the organism and could be used for further inoculation. He inoculated as many as fifty mice and never encountered a case of natural immunity. The organisms stained readily with any of the ordinary stains—Giemsa, fuchsin, methylene blue, etc.

"In stained preparations the spirochete is seen as a uniformly staining spiral; the longer forms, however, show a clear unstained central spot (see Diagrams Nos. 8 and 9). The ends are slightly tapering; there is no sign of a nucleus or undulating membrane. As just mentioned, the larger forms have a clear spot at their center, and in some of these the body of the spiral tapers toward this spot. In some cases two small spirals are attached end to end by an unstained region. These forms are evidently stages of transverse division. No indications of longitudinal division were seen nor any mode of reproduction other than the one just mentioned.

"The number of the turns of the spiral varies from six in the longest forms to two in the shortest. The lengths of the spirochetes vary from 6 or 7 μ to 3 or 4 μ. The width is about 2 μ.

"The spirochete was always seen in the spiral form; no other forms were found at any time."

Wenyon states that, after discussing the matter with M. Borrel, they arrived at the conclusion that the organism just described and the one observed by Borrel[2] in the juice of malignant growths of mice are the same organism; comparison of Borrel's preparations and those of Wenyon lead to the same conclusion. Wenyon attempted to test the theory of Borrel that these organisms had migrated into the tumors from the intestinal tract, by inoculating mice with mucus from the intestinal tract of mice which contained large numbers of varying kinds of spirochetes. These conclusions were negative; no infection or abscess resulted. The mucus from the intestinal tract of a mouse infected with *Spirochaeta m.* likewise gave negative results. Wenyon concluded that there is no evidence that these spirochetes have originated in the intestine.

The description given by Wenyon of the organism found in the blood of otherwise normal mice clearly distinguishes it from the organism which we have described. If it be identical with the organism found by Borrel in his mouse tumors that would seem

[1] *Jour. Hyg.*, 1906, 6, p. 580.

[2] *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 770.

to distinguish this organism from the organism which we have found. Borrel does not give the method by which he succeeded in demonstrating the organism found in his mouse tumors, but as his publication antedates that of Levaditi, it is highly probable that it was by means of the Giemsa or some aniline staining method. Whether our inability to stain our organism with any of the aniline methods will prove a means of distinguishing it from other small organisms, such as that of Löwenthal and the smaller organisms found by Borrel in combination with helminthia, remains to be seen, but the forms with the coarser gyrations appear to be clearly distinguishable from our organism.

The distribution of the organisms through the primary tumors examined is of great importance. A careful study of these tumors will appear to throw some light on whether or not the organisms were accidental in their relation to the tumors in which they are found, or whether they may bear some etiological relation. A description of one of these tumors will suffice for all, the only difference being in the number of organisms which the small fragments impregnated with silver happen to contain. The greatest number were found in the primary tumor known as G-7, and, with the qualification that the other tumors contain fewer organisms, the description of this tumor will apply to the remaining eight.

This tumor was sent from Massachusetts and remained in the laboratory about two days. The tumor was about the size of a small bean, measuring approximately 1 cm. by 0.6 cm. in its greatest diameter. It was slightly flattened, freely movable beneath the skin, and was situated on the ventral aspect. The mouse was killed and the tumor removed September 20, 1906. Weight of mouse 33.5 grams, weight of tumor 1.5 grams. Six mice, Lot 1031, were inoculated with portions of the tumor. Results to date negative. Small fragments of the tumor were hardened in formalin, and some of them impregnated by the Levaditi method.

An examination of a section of the tumor hardened in formalin and stained with Borrel's method, with low power, shows the tumor to be a rapidly growing adenocarcinoma of the breast. In many portions the tumor assumes the characteristics of a rapidly growing carcinoma solidum. At the edge of the tumor is the usual characteristic proliferation of the connective tissue invaded by prolongations and nests of epithelium from the tumor mass. In some portions of the periphery a more or less definite connective tissue capsule is present. Scattered through the tumor are the small areas of necrosis which characterize rapidly growing tumors of this type. The stroma is in some portions of the tumor poorly developed, in others well defined. Here and there, especially at the margins where the tumor is penetrating into the connective tissue structure, the tendency to the adenomatous type is quite marked. In these

regions cysts of considerable size can be found, filled with the usual coagulated fluid mixed with cell detritus. A careful examination of such a section with oil immersion fails to show the presence of any organisms, even after comparison with sections from the immediate neighborhood impregnated with the Levaditi method in which plentiful spirochetes are present. Not even unstained structures which one might identify can be determined.

Sections from small pieces of the tumor impregnated by the Levaditi method, examined under high power, present a very striking appearance. First of all, lying free within the cysts in the coagulated contents, are found sharply defined, intensely black spirals, measuring from 2.5 to 7.8 μ in length. The corkscrews are from three or four to 13 or even more in number. These organisms are more or less regularly distributed through the cyst spaces (see Fig. 4, Plate 3), and present so characteristic an appearance that they can, to our minds, never be confused with nerve endings. In the rapidly growing portions of the tumor between the epithelial cells one frequently encounters aggregations of five or six, or even a dozen, of these characteristic organisms lying in small, clear spaces between the epithelial cells, as though they were surrounded by some clear fluid producing a form of vacuole (Fig. 6, Plate 3).

Passing to the margins of the tumor, one finds that in the nests of epithelium penetrating into the connective tissue stroma the lumena of the small tubular structures frequently contain aggregations of organisms, and here and there free epithelial cells with organisms in their protoplasm. One can easily imagine that the development of the larger cysts in the tumor might be determined by the proliferation of the epithelium around the small groups of organisms thus found. Beginning with a secretion of fluid around the organisms, as shown in Fig. 6, Plate 3, the proliferation of the epithelium might produce the beginning of a cyst, such as is represented by Figs. 8 and 3; and the continued secretion of fluid and associated proliferation might ultimately terminate in the development of a larger cyst, such as Fig. 4. The invariable presence of organisms in the larger cyst cavities would suggest such a possibility. The organisms are distributed in this tumor in such a way that the largest numbers of organisms are in the most rapidly growing portions of the periphery. Occasional organisms are found in the connective tissue beyond the growing edge of the tumor. This is especially well seen where the epithelium is penetrating into the adjacent fat tissue. Here organisms will be found between the fat cells a short distance in advance of the epithelium, but when one investigates the surrounding normal tissues beyond this zone, no organisms are to be found. Where the stroma of the tumor is best developed one frequently finds organisms in the stroma. Where this is not so well defined, they lie between the epithelial cells, as shown in Figs. 3, 6, and 8, Plate 3. Occasionally organisms can be found within the epithelial cells. Here they are frequently curved into ring forms (Fig. 7, Plate 3), or in aggregations suggesting agglutination (Fig. 7, Plate 3). The characteristic spiral form of the organism is not always apparent. Occasional forms will be met where the silver appears to have impregnated the organisms homogeneously. This is usually the case with organisms within the protoplasm of epithelial cells (Figs. 7 and 8, Plate 3), and in these cases the organism frequently presents a beaded appearance suggestive of involution forms or degeneration approaching disintegration. In regions where phagocytosis is active one will find fragments of the organisms in the protoplasm of the epithelium, the organisms in these cases appearing to break up into minute granules or short S-shaped structures

similar to the changes in *Sp. Obermeieri* in mononuclear macrophages in the peritoneal cavities of hyperimmunized rats (Pfeiffer's phenomenon).

The examination of sections of the remaining eight carcinomata of the breast in mice differ in no way from the findings in G-7. In the tumors in which there are few organisms these are found exclusively at the very margin of the growing edge, in the center of cell nests, and in the connective tissue stroma surrounding the tumor.

In the transplanted tumors the distribution is even more pronouncedly at the margin of the tumors. In rapidly growing, early transplants, the organisms are massed in the zone of connective tissue proliferation at the margin, and in the more virulent tumors, such as the Brooklyn tumor, the organisms are frequently present in great numbers (Fig. 5, Plate 3). Involution forms and disintegrating organisms are very frequent in these areas. Phagocytosis does not seem to be so active in these tumors. Careful examination of the sections from the Springfield, Jensen, and Brooklyn strains shows no differences in appearance or measurements, distribution, or characteristics of the organisms in the different strains. We appear here to be dealing with a definite organism. In large, late transplanted tumors, in which there are areas of extensive necrosis, the organisms are exclusively confined to the growing margins of the tumor. When, as occasionally happens, the necrosis extends to the periphery of the tumor, the adjacent connective tissue zone contains either no organisms or occasionally disintegrating or involution forms. In two large tumors examined, which were badly contaminated with bacteria, no spirochetes whatever could be found.

With the determination of the practically constant association of this characteristic organism in primary and transplanted mouse tumors naturally arises the question as to their significance. Although it will require a prolonged and careful search through other types of tumors, especially in other animals and in human beings, before the relation of this organism to the tumors in question can be definitely ascertained, still it would seem not improper to discuss in the light of the striking evidence of cage infection in this very group of tumors, the possible etiological significance of these organisms. Up to the present time those who have sought for parasites in cancer have searched for an intracellular organism, and at first glance it would

seem difficult to understand how an organism with the distribution of the one in question could be responsible for the epithelial proliferation necessary for the production of a malignant growth. We have for some time assumed that exrtacellular organisms might be the cause of the proliferation of epithelium through the medium of some toxic substance which they elaborate. Evidence of the existence of some bio-chemical substance of this sort in tumors has come to us in the course of our experimentation. The interesting work of Clowes and Baeslack from this laboratory on "The Influence Exerted on the Virulence of Carcinoma in Mice by Subjecting the Tumor Material to Incubation Previous to Inoculation," is a case in point, Clowes having reasoned that the best explanation of the marked increase in virulence produced by a short period of incubation resulted from the effect of increased temperature upon the rate of reaction of some stimulating substance contained in the tumors. These interesting observations have had a very positive and new light shed upon them by the work of Bernhard Fischer.[1] Fischer has made the discovery that there are certain chemical substances (thus far the fat stains, scarlet R, Sudan III, and indophenol) which possess a positive chemotactic quality for the epithelium of the skin in rabbits, and possibly some other animals. This chemotactic or attractive quality is exerted only when these fat stains, dissolved in olive oil, are injected into the subcutaneous tissue beneath the skin. Here they set up a chronic connective tissue proliferation similar to that found about the margins of beginning epitheliomata of the skin. The scarlet R oil penetrates into all the lymph spaces and crevices of the tissue and, with the advent of the chronic connective tissue proliferation, exerts a chemotactic attraction for the epithelium of the deeper layers of the adjacent skin. Oil alone does not exert an activity to this extent, and it is Fischer's opinion that part of the scarlet R, although but slightly soluble in water, is taken up by the lymph and transplanted to the epithelium which it stimulates to proliferation. Within a short period (three weeks) the presence of the scarlet R in the subcutaneous tissue produces an active proliferation of the epithelium of the skin, of the hair follicles and sebacious glands, associated with the presence of typical and atypical karyokinetic figures. The epithelium pene-

[1] *Münch. med. Wchnschr.*, 1906, 53, p. 2041.

trates into the surrounding connective tissue in the form of charac teristic prolongations and nests, such as characterize the beginning of squamous epithelioma of the skin. Typical epithelial pearls are formed, and Fischer states that at this stage of the process the histological appearance is indistinguishable from epithelioma of the skin. The illustrations which he gives confirm this opinion. That it is the scarlet R which attracts the epithelium is shown by the epithelium growing down to, surrounding, and gradually removing the saturated oil drops. Where the scarlet oil penetrates into the lymph spaces the epithelium proliferates in the lymph spaces in pursuit of it, and in one case, where Fischer wounded the cartilage with the needle used for injection, he found the epithelium penetrating into the lymph spaces of the cartilage and the clefts which contained the scarlet oil. That part of the coloring matter is diffused through the tissues; and that it is in this way that scarlet R produces its first effect upon the more or less distant epithelium is shown by the occasional extensive staining of the fat constituents of the adjacent cartilage cells where the scarlet R has been injected into the connective tissue in the immediate neighborhood. Fischer found that scarlet R affects only the epithelium of the epidermis of the rabbit. Attempts to produce similar proliferations by injecting the stain into the breast, under the epithelium of the stomach and intestinal tract, have proven invariably negative. In one case of a dog in which a large amount of scarlet R was injected beneath the skin producing the characteristic proliferation of the epithelium, small nodules apparently derived from proliferation of the alveolar epithelium of the lung were found, and Fischer suggests that possibly this type of epithelium may prove less specific than others and also react to the stimulus of scarlet R.

From his experiments it would appear that scarlet R is capable of affecting only one kind of epithelium, the epidermis. Upon the cells of this structure it exerts, from its position in the subcutaneous tissue, an attractive or chemotactic function which causes the epithelium to proliferate in the deeper structures, producing in the height of its activity, a picture indistinguishable from beginning carcinoma. Here, however, the analogy ceases; for when the scarlet R is entirely absorbed by the epithelium, the cells rapidly hornify,

and the entire process subsides. Fischer points out that all that is needed to extend this process into carcinoma would be the local, continuous production of some chemical substance similar in its affinities and characteristics to scarlet R. In a footnote he states that he appreciates that the advocates of the parasitic theory can utilize his observations in support of some parasite working through the medium of an *attraxine*, as he calls this substance; in which case it would be necessary, however, to assume a special organism for every type of epithelium subject to cancerous transformation.

The distribution of the organism which we have described corresponds in very striking degree to the distribution of the scarlet R in Fischer's experiments. The absorption and removal of the scarlet oil by the epithelium finds its counterpart in the evidence of phagocytosis which we have noted. If the spiral organisms found in our tumors are the cause of these tumors, then they produce the proliferation of the epithelium through the medium of some toxic substance which they elaborate. That such a toxic substance possibly exists has, as we have pointed out, already been shown by Clowes. To our minds, the necessity of assuming a different organism for each type of epithelium rather simplifies than complicates the problem, and it is not impossible that organisms belonging in the same class, or of widely different characteristics, may possess the power of elaborating specific toxic substances. A striking possibility in this connection is the so-called Bilharzia disease, in which typical cancer of the bladder is associated with the presence of the embryos of the worm in the bladder wall. It is also of interest that in Bilharzia disease no metastases have ever been found, although the local disease of the bladder presents the characteristics of infiltrating carcinoma.

The evidence of immunity associated with these tumors is, to our minds, likewise suggestive of a possible etiological significance of these organisms, and, in this connection, the conditions found in the tumor 3^{10} referred to at the beginning of this article are of great interest. This tumor at the time of its removal and hardening had begun to retrograde and presented the histological characteristics of spontaneously retrograding tumors. It is possible that this tumor was hardened at the moment when a very active phagocytosis on the part of the remaining epithelium was in progress. As shown in Fig. 1,

Plate 3 of this article, and in Figs. 5 and 6, Plate 17 of Calkins and Clowes's article in this journal, many of the epithelial cells of this tumor contained large numbers of small, rodlike structures, which, in the writer's opinion, are the organisms we have described, incrusted with mercury. If one studies the vacuoles containing these structures in sections of this tumor, one can trace through the smaller vacuoles a gradual disintegration and final disappearance of these structures within the vacuoles. The whole presents a picture which strongly suggests the description given by Novy and Knapp[1] of the disintegration of *Sp. Obermeieri* in the bodies of phagocytes after the injection of blood containing large numbers of this organism into the peritoneal cavity of recovered rats. As the entire process in the case of *Sp. Obermeieri* occurred in a period of less than 10 minutes, it would appear that, if the appearance found in this tumor illustrated such a phenomenon in the mouse tumors, the tumor was placed in the hardening agent at the psychological moment. It will, however, require further experimentation to confirm the significance of these appearances.

It is obviously too early to draw far-reaching conclusions from the presence of this organism in our mouse tumors. First of all, more extensive experiments must be carried out. These are in progress, and the organism is being studied in all its relations to the tumor in question, in which investigations Drs. Calkins and Clowes have joined. The description of what appear to be similar organisms impregnated with silver in a case of uncontaminated human cancer of the breast by Friedenthal naturally suggests that a further search for these organisms in human tumors may lead to positive results in this connection, and such a systematic search has already been inaugurated. At present it appears to us that the constancy with which this organism is found in the primary and transplanted mouse tumors is very striking; that in the light of Fischer's work it would appear to us quite possible that an extracellular organism distributed as this organism is distributed, could be the cause of the proliferation; that the evidence of phagocytosis and the detection of these organisms at times within the protoplasm of epithelial cells would sufficiently explain the establishment of metastases by the transportation of

[1] *Jour. Infect. Dis.*, 1906, 3, p. 291.

infected cells. The organism should be found with continued regularity, in which case it would appear to bear the same relation to these tumors as does *Sp. pallida* to syphilis.

SUMMARY.

1. In 1905 there were found in vacuoles in the epithelial cells of a retrograding mouse tumor, fine, rodlike structures impregnated with mercury. These the writer held to be parasites.

2. Attempts to fix these structures were not successful until the advent of Levaditi's method. With this method a characteristic spiral organism 2.5 to 7.8 μ in length and 0.6 μ in width, with 4 to 13 turns per individual, has been demonstrated in 10 consecutive spontaneous carcinomata of the breast in mice obtained from Massachusetts, Ohio, and New York. In one other tumor the hardening method was a failure.

3. An examination of sixteen transplanted mouse tumors from three different sources shows the presence of the same organism in all tumors examined.

4. An examination of fresh materials from all transplanted uncontaminated tumors from these strains demonstrates the organism in the living state. It is frequently motile and is found with difficulty.

5. Measurements made on the fresh organism correspond closely with the measurements made in the stained preparations.

6. The distribution of the organism through the primary tumors shows that they are most prevalent in the most actively growing portions of the tumor; that they live in the connective tissue at the margin of the tumors, and in the stroma of the tumor; that they are found between the epithelial cells of the tumor and in the cyst cavities of the tumors where these are present.

7. In the early transplanted tumors the organisms are found in the connective tissue zone at the growing edge and between the cells at the growing edge.

8. The more virulent tumors contain the greatest number of organisms.

9. In both primary and transplanted mouse tumors evidences of phagocytosis on the part of the epithelial cells are to be found. The organism in these cells frequently assumes the form of rings and breaks down into small S-shaped segments and granules.

10. In two tumors badly contaminated by bacteria no spirochetes could be found.

11. The examination of organs and subcutaneous tissues of five normal mice by the Levaditi method has shown no spirochetes.

12. All attempts to stain the organism with aniline stains to date have proven unsuccessful.

13. The organism is morphologically distinguished from the organism described by Wenyon in the blood of mice.

14. It would appear to be distinguishable from the *Spirochaeta microgyrata* of Löwenthal and the organisms found by Borrel in mouse tumors, both of which are stained with the Giemsa stain, by its inability to take this stain.

15. Our observations do not as yet establish an etiological relation between this organism and cancer of the breast in mice, but the presence of the organism in primary mouse cancers with which it is regularly transplanted through many generations, greatly increasing in number as the tumors increase in virulence, instead of interfering with, and finally preventing, transplanation as do bacteria, is suggestive.

DESCRIPTION OF PLATE 3.

FIG. 1.—Epithelial cell with large vacuole in protoplasm. Nucleus pushed to one side. Vacuole filled with fine rodlike bodies some of which show gyrations and beadlike structure. Taken from Jensen mouse tumor 1905. Sublimate fixation. Organism unstained. ×1,030.

FIG. 2.—Spirochetes from margin of transplanted Springfield tumor. Carcinoma solidum of the breast. Levaditi silver method. ×1,360.

FIG. 3.—Spirochetes between the epithelial cells of primary adenocarcinoma of the breast in mouse G-7. ×1,030.

FIG. 4.—Section through cyst in carcinoma of the breast, mouse G-7, showing three spirochetes in cyst contents. ×1,030.

FIG. 5.—Large numbers of spirochetes in margin of rapidly growing transplanted carcinoma of the breast, Brooklyn tumor. ×1,030.

FIG. 6.—Group of organisms between the epithelial cells of primary carcinoma, mouse G-7. ×1,030.

FIG. 7.—Spirochetes in protoplasm of epithelial cell, same tumor as Figs. 3, 4, and 6, showing phagocytic action of epithelium. Spirochetes in form of a ring and agglutinated organisms. ×1,030.

FIG. 8.—Spirochetes in protoplasm of ephithelial cell at the center of nest of growing epithelium. ×1,030.

In Figs. 1, 3, 4, 5, 7, and 8 the spirals are not easily seen, owing to the low magnification which was chosen to show the relation of the organisms to the tissue. In Figs. 2 and 6 the spirals are perfectly distinct. In every case, however, except in Figs. 7 and 8 the spirals are plainly discernible through the microscope. In Figs. 7 and 8, representing degeneration forms, the spirals are not intact.

PLATE 3.

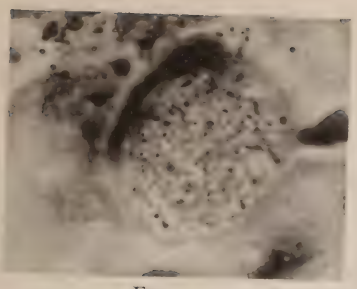

FIG. 1.

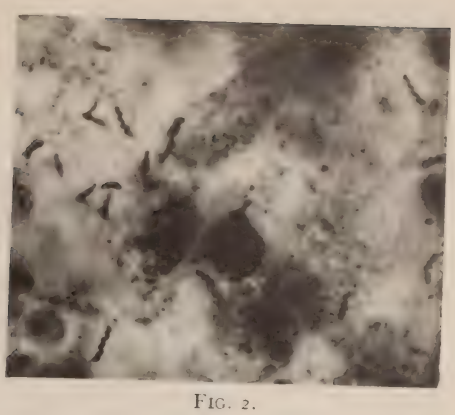

FIG. 2.

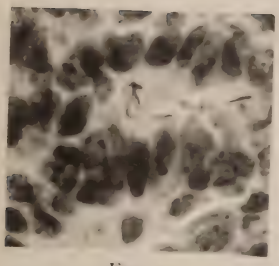

FIG. 3.

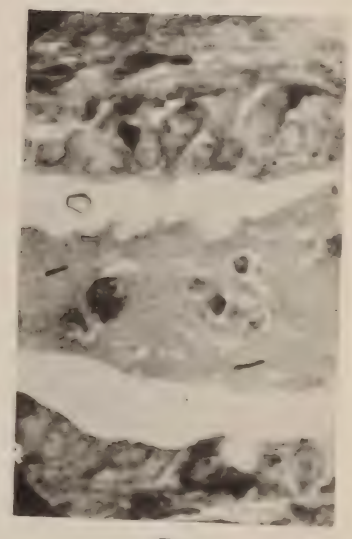

FIG. 4.

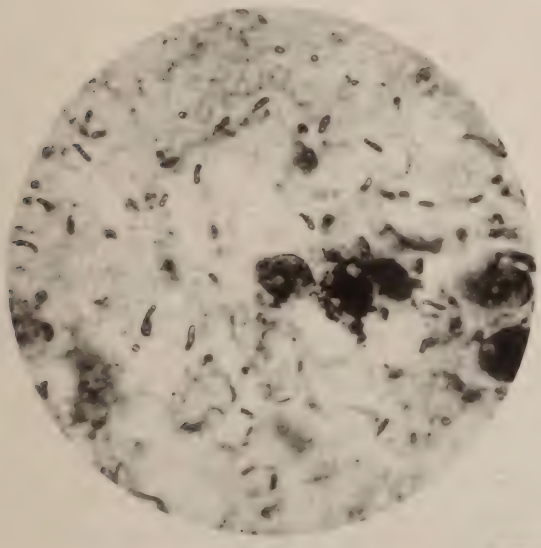

FIG. 5.

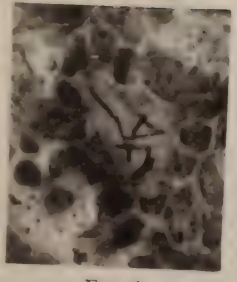

FIG. 6.

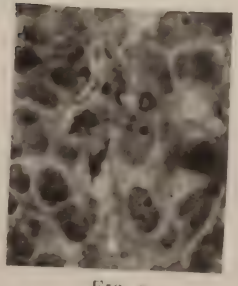

FIG. 7.

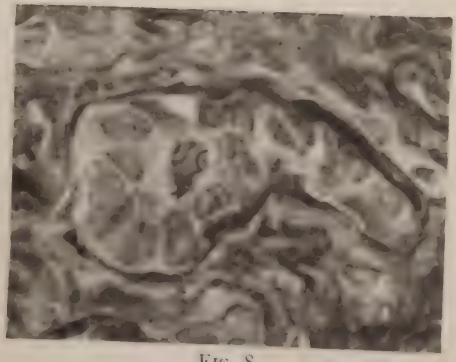

FIG. 8.

A SPIROCHETE IN MOUSE CANCER, *SPIROCHAETA MICROGYRATA* (LÖWENTHAL) VAR. *GAYLORDI*.*

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WITH the uncertainty at the present time which attaches to species of *Spirochaeta*, it does not seem wise to make a new one for this parasite of the mouse tumors. Both in the case of *Trypanosoma* and of *Spirochaeta* new species have been created on the basis of purely physiological grounds, and many of the supposed "good" species of both of these genera will probably turn out to be mere variants of one or another. The mere fact that an organism will not live in the blood which serves as a culture medium for another organism of apparently identical structure and character is not enough to satisfy the requirements of a new species. There are many species of protozoa which are represented by salt- and fresh-water forms, and although the fresh-water forms cannot live if placed in salt water, nor salt-water forms in fresh water, no one would think of making different species of these organisms because of their mode of life. So it is with these spirochetes and similar flagellates; a physiological difference, while important in matters of therapeutics, has little value in taxonomy, and the creation of new species therefore may well be left until the life-history of different forms is made out.

Thus it is with the spirochete in mouse cancer which Gaylord discovered last summer. In dimensions and in general character the organism agrees perfectly with the one which Löwenthal described in 1906 in ulcerated human carcinoma, in a dog tumor, and in feces; but his spirochete stained readily with the Giemsa stain, and the present one stains with the greatest difficulty. It will be remembered that this difference was the sole distinction between *Sp. refringens* and *Sp. pallida* at the time of their discovery, although subsequent observations have shown that the one has an undulating membrane and no flagella, while the other has flagella and no membrane. In view of this difference in staining capacity

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and in the host of the organism on the one side, and for convenience in reference in future work on the other, I venture to give to this parasite in mouse cancer a new variety name, with the understanding that, should future research show it to be a distinct species, the variety name shall become the specific name. Löwenthal's species name *Spirochaeta microgyrata* is sufficiently distinctive to characterize the organism, and to this I would add the variety name *Gaylordi*, in recognition of the fact that Dr. Gaylord was the first to study the relation of this organism to these tumors.

With the exception of Löwenthal's original description,[1] there has apparently been no subsequent recognition of this species. We may have seen it in 1905 as the basis of the curious artefacts which appeared in one of the mouse tumors at the Buffalo laboratory, but it was certainly not recognized at that time as a spirochete. Borrel[2] may have seen it in 1905 in the three mouse tumors in which two forms of spirochete were observed, but no measurements and no figures were given, and no importance was attached to the presence of the organisms. Wenyon[3] evidently did not see it in his *Spirochaeta muris*, for the dimensions which he gives belong to an altogether different organism.

There is considerable question as to what constitutes the "unit" organism in *Spirochaeta*, and the majority of specific characterizations in consequence are vague and misleading. Most observers give the number of "turns," or, as I shall call them, "nodes" (a node representing the part from the crest of one undulation to the crest of the next adjacent), and the total length, both of them varying within rather wide limits. Such wide variations, especially when the organisms are supposed by many to divide by longitudinal division, are incompatible with what we know of unicellular forms generally, and the a priori conclusion is that these diverse sizes are the result of end-to-end union of smaller units forming variable aggregates which recall other catenoid colonies among the protozoa. Löwenthal, Krzysztalowicz and Siedlecki, Schütz, and others describe the breaking-up of spirals of *Sp. pallida* into smaller elements which are variously intrepreted, while Novy describes the fragmentation of *Sp. Ober-*

[1] *Berl. klin. Wchnschr.*, 1906, 43, p. 283.

[2] *Compt. rend. de la Soc. de Biol.*, 1905, 58, p. 770.

[3] *Jour. Hyg.*, 1906, 6, p. 580.

meieri under the action of phagocytes. These observations afford some evidence for the view that the unit *Spirochaeta* is much smaller than the forms ordinarily seen.

FIG. 1.—*Spirochaeta microgyrata* Löw., var. *Gaylordi*. A single specimen from a transplanted tumor of the Jenson series. × 2,500.

At the present time, and until the life-history is known, the most satisfactory basis for identification of the spirochete of mouse cancer is the measurement of the single node. This may be determined either by direct measurement of the node or by ascertaining the total length of the organism and the number of nodes, and thus getting the average size of the node. As it ordinarily appears, the organism measures from 2.5 μ. to 7.8 μ in length, and it is made up of from 4 to 13 nodes. The average length of the node in a great many measurements is 0.6 μ, and the organism is 0.6 μ wide. The nodes therefore are characteristically crowded and the undulations steep. Compared with *Sp. Obermeieri*, *Sp. refringens*, and *Sp. pallida*, this form is much shorter, has more crowded nodes and a greater diameter both relatively and absolutely (Fig. 1).

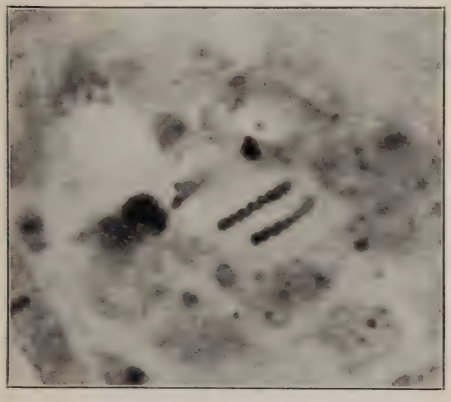

FIG. 2.—A group of spirochetes (two in focus) from a primary tumor from the zoological laboratory, Columbia University. × 2,500.

The ends of *Spirochaeta microgyrata Gaylordi* are blunt and rounded and there is no evidence of tapering at the extremities, nor of undulat-

ing membrane, nor of flagella. (The apparent flagellum seen in Fig. 1 is an illusion caused by a slight fold in the membrane of the host cell in which the spirochete lies.)

In this preliminary communication it will not be possible to describe all of the changes which the spirochete undergoes in the cancer cells. Some of the changes that have been observed are undoubtedly phases in development of the organism as ordinarily seen, while others are undoubtedly involution forms. These different conditions are best seen in primary tumors, several of which have been obtained at the Buffalo laboratory and one at Columbia University in a mouse that had not been near the Buffalo laboratory, nor had it been near mice or cages from that place. I state this in some detail because of the possible criticism that spirochetes of this same type are found in all the tumors at the Buffalo laboratory because of a widespread infection there. Fig. 2 is a photograph of spirochetes from the primary tumor that developed in the Columbia laboratory, and Fig. 3 is a photograph from the same tumor showing a reduced condition of the spirochetes, perhaps due to the phagocytic action of the cancer cells.

FIG. 3.—Two individuals undergoing degeneration. From the Columbia tumor. × 2,500.

Various enigmatical structures are found in the nuclei of the epithelial cells of these primary cancers—structures which I have failed to find in any of the normal glandular tissue which has been treated in the same way as the cancer tissue (i. e., by the Levaditi silver impregnation method). For this comparison I have examined carefully tissue from thyroid, liver, intestine, and, in addition, mesentery connective tissue and muscles, all from normal mice, and in no case have I found the organism or intranuclear bodies mentioned above. In a future publication I hope to be able to throw some light on the significance of these intranuclear structures.

PURPURA HEMORRHAGICA WITH GENERALIZED INFECTION WITH *BACILLUS PARATYPHOSUS*.*

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Opinions are at variance as to the propriety of recognizing a new disease, "paratyphoid fever," in certain infections with *Bacillus paratyphosus*.[1] The chief reasons for doing so at present are the agglutinative properties of the blood serum and the results of bacteriologic investigations of the blood during life and of the body post-mortem. Skepticism concerning the value of agglutinative reactions of the blood serum as the only reliable means of recognizing the occurrence of such infections is found in the writings of Jürgens, Drigalski, Grünberg and Rolly, Conradi and Kayser, and others;[2] furthermore, since the anatomic entity established by Wells and Scott[3] considerable evidence has accumulated of the occurrence of *Bacillus paratyphosus* as an etiologic factor in food poisonings, Trautmann[4] especially maintaining that paratyphoid fever is a subacute meat poisoning.[5] It would seem best perhaps, for the present at least, to concede to the paratyphoid infections more of etiologic than of anatomic or clinical entity, pending the accumulation of an adequate number of carefully studied cases to permit of satisfactory nosologic classification. The case reported here offers some features of exceptional interest.

Clinical history.—The patient, a Canadian woman, 36 years of age, married 10 years, entered the Presbyterian Hospital on May 21, 1906, to the service of Dr. Bevan, to whom we are indebted for the opportunity to study and report the case. For over a month she had suffered from drowsiness, depression, and indisposition to work, and during this period experienced a regular evening rise of temperature which at times reached 103° F. Her appetite was poor, and for 10 days she was troubled with a dry cough. There was some tenderness over the abdomen, occasionally becoming localized in the left iliac region. The attending physician reported that two examinations of the blood serum had been made for its agglutinative action on typhoid bacilli,

* Received for publication February 12, 1907.

[1] Ascoli, *Zeitschr. f. klin. Med.*, 1903, 48, p. 418; Kolle, *ibid.*, 1906, 52, p. 285.

[2] See Boycott, *Jour. Hyg.*, 1906, 6, p. 33.

[3] *Jour. Infect. Dis.*, 1904, 1, p. 72.

[4] *Zeitschr. f. Hyg.*, 1904, 46, p. 68.

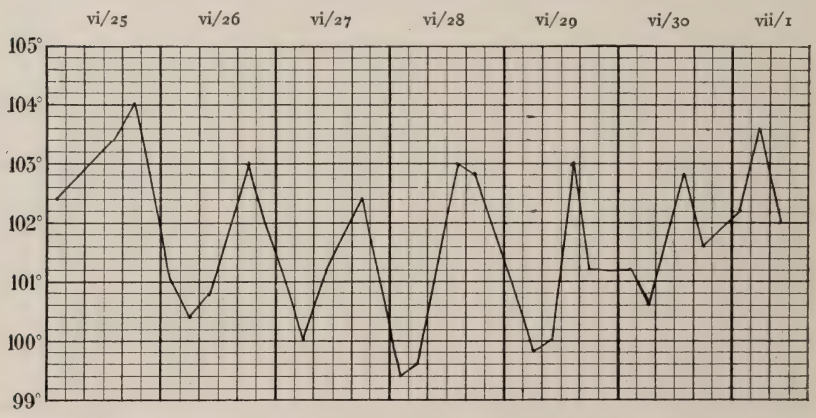
[5] See also the report by Vagedes, *Klinisches Jahrbuch*, 1905, 6. p. 517.

but in neither had it showed any such properties. During this illness she had lost 25 pounds in weight.

Examination at the time of admission into the hospital revealed a few enlarged cervical glands, a coated tongue, some tenderness in the left hypochondriac region, a high pulse, and a variable temperature. The blood seven days after entrance showed 20,500 leucocytes, hemoglobin 60 per cent. The urine contained a trace of albumin and a few cells thought to be leucocytes.

She left the hospital on May 25 and for three weeks tried outdoor living and forced feeding, but became steadily weaker and lost in weight. Her temperature every evening was high, and the latter two weeks of this period she was troubled with frequent and painful urination. Her pulse ranged from 90 to 120. Shortly after she left the hospital a rash appeared on the neck and chest, with bluish or purple blotches on the legs, arms, chest, and abdomen. The bowels were sluggish, and she had epistaxis frequently.

Fig. 1.—Temperature Curve. June 25 to July 1.



When she returned to the hospital she was pale, the conjunctival vessels engorged; there was a bluish-red spot 2 by 4 mm. in the conjunctiva near the left pupil, and a similar but larger spot on the bulbar conjunctiva of the right eye; the cervical glands were enlarged. There were discrete, closely set, light-brown, slightly elevated spots, 2 to 3 mm. in diameter, on the neck and abdomen; likewise purplish ecchymoses on the extremities, chest, and abdomen. The abdomen was tympanitic and tender in various regions.

Examination of the blood on June 25 showed 3,050 leucocytes, hemoglobin 60 per cent; on June 27, 9,450 leucocytes, 2,800,000 erythrocytes, and hemoglobin 47 per cent. The urine was cloudy, reddish brown in color, neutral in reaction, with a specific gravity of 1025, and no odor; it contained albumin, leucocytes, and erythrocytes. The temperature from June 25 to July 1 is shown in the accompanying curve. She was unable to eat, restless and wakeful, and complained quite frequently of pain in the abdomen and severe pain on urination. The stools were large in amount, brown in color, and of semi-solid consistency. She vomited occasionally and had frequent epistaxis. Rectal food was retained quite well, but occasionally expelled, and on these

occasions was tinged red with blood. She became very weak, and on June 30 was delirious, seemed to be in great pain, and died on July 1.

Anatomical changes.—At the postmortem examination by Dr. Rosenow one hour after death the following changes were found: Hyperplasia of the intestinal lymphoid tissue; hyperplasia of the mesenteric, retroaortic, and iliac lymph glands; acute splenitis; subcutaneous and submucous hemorrhages in the urinary tract; renal and urinary calculi; cloudy swelling of the liver and kidneys; fatty liver; sordes; recent and ancient pelvic peritonitis; hydro- and hemato-salpinx; hemorrhages in the right ovary; horseshoe kidney; accessory spleen; sclerosis of the aorta and coronary.

"The body is 135 cm. in length, the abdomen is rather full, though not distended. Brownish spots are present over the anterior surface; on the right breast they are more purple and measure here from 1 to 4 mm. in diameter. Some of the spots are recent subcutaneous hemorrhages and measure 1 to 4 cm. in diameter. The largest are on the right side mainly, the arm, forearm, breast, hip, and thigh. The lining of the mouth shows no changes."

Deserving of special attention are the changes found in the intestinal glands and the urinary tract.

"The lymphoid tissues in the small intestine are slightly enlarged, the solitary follicles not exceeding 1.5 mm. in diameter. Very slight erosions of their surfaces and of the surfaces of the Peyer's patches can be seen on close scrutiny. By holding the small intestine to the light the Peyer's patches appear to contain more blood than the adjacent wall. The large intestine shows no changes. The mesenteric and peripancreatic lymph glands are all enlarged, some measuring 3.5 cm. in their longest diameters, firm and light gray on the cut surface. In the iliac and periaortic glands, also markedly enlarged, hemorrhages are present, and these glands are softer."

Some glands about the aorta were so full of blood that they were thought to be hemolymph glands by Dr. Rosenow.

"The spleen, which is soft and has all the characteristics of an acute infectious disease, weighs 260 grams. On the surface exposed by sectioning the Malpighian bodies are not especially distinct. A small accessory spleen shows a similar appearance.

"In the kidney, which is 'horseshoe-shaped,' there are extensive hemorrhages in the mucous lining of the pelvis. On the cut surface the kidney is mottled with gray and grayish-red areas; the cortex appears swollen. In the upper part of the pelvis are some soft, small calculous masses. Similar submucous hemorrhages are much more marked in the bladder, which also contains soft, small calcareous masses similar to those in the kidney. The hemorrhages are so excessive that but little of the more normal pale mucosa is visible.

"In the right ovary the contents of two cysts, together the size of a hen's egg, appear to be almost pure blood; there is no clot, and the linings are smooth. The outer end of each Fallopian tube is closed by the fibrous adhesions, which are abundant in the small pelvis. Both are distended, the contents of the right are clear, of the left chocolate-colored."

Bacteriological examination.—A short, actively motile bacillus, decolorized by Gram's method, was obtained from the blood during life, and postmortem from the spleen, pelvic lymph glands, heart's blood, peritoneal fluid, left tube, and from the bile. *Staph. pyogenes aureus* was isolated from the pericardial fluid, but was not present in the other material examined.

On agar plates the superficial colonies of the bacillus appeared as circular spots

of a dull gray color by reflected light and bluish gray by transmitted light. They were smooth and moist, with finely crenated edges. The deep colonies were generally spherical and more opaque than the superficial ones. On potato the bacillus grew well after one or two days, the cultures being smooth, raised, moist, bluish white in color, without odor and discolorations. On blood serum it grew well, the growth being white, raised, moist, causing a white turbidity of the water of condensation. In broth a uniform cloudiness appeared in 24 hours, and gradually a white precipitate settled out which was easily diffused again. On gelatin there was a thin white growth along the line of inoculation, but no liquefaction. In glucose-agar stabs gas was formed in 24 hours which obscured the characteristics of the growth. In litmus milk there was at first a slight acidity, which changed in five days to alkalinity, which gradually became more pronounced and permanent. There was no coagulation at the end of four weeks. Indol tests gave negative results. Neutral-red agar turned yellow near the center in 24 hours, and this yellow color gradually extended to the bottom of the tube, but never to the surface. In nine days the yellow color disappeared, and the medium returned to its red color.

By growth in fermentation tubes the following gas formulae were obtained for the carbohydrates indicated:

Glucose*..... $\frac{8.5}{2.5}$	Amylose .....a trace	Lactose* ..... $\frac{0}{0}$
Maltose*..... $\frac{8.5}{3.5}$	Arabinose .....a trace	Nutrose*..... $\frac{0}{0}$
Galactose*..... $\frac{7.5}{2.5}$	Dextrin .....a trace	Inulin*..... $\frac{0}{0}$
Saccharose*.... $\frac{4}{1}$	Isodulcite .....a trace	Raffinose..... $\frac{0}{0}$
Mannit*..... $\frac{4}{1}$		Mannose ..... $\frac{0}{0}$
Melitose..... $\frac{5}{1}$		
Levulose..... $\frac{5}{1}$		

*Repetition twice showed the same results.

The serum of a rabbit immunized by injections of broth cultures of the Buxton paratyphoid bacillus,[1] seven injections in a month, agglutinated this bacillus in a dilution of 1 to 1,000 in 20 minutes, the organism from the Wells-Scott case in a similar dilution in 30 minutes. Immune serum obtained with the Wells-Scott organism[1] agglutinated this organism in a dilution of 1 to 1,000 in 40 minutes and the Buxton bacillus in 45 minutes. The immune serum of a rabbit injected with the bacillus from this case over a period of two months and at intervals of four to seven days, agglutinated the Buxton bacillus more strongly than the Wells-Scott organism—the former in 1 to 1,000 in 40 minutes; the Wells-Scott organism in a dilution of 1 to 900 in 70 minutes.

All the immune sera naturally agglutinated the organisms inoculated; none of them the typhoid bacillus.

Microscopic anatomy.—The intestinal glands are spread apart by hyperplasia of the lymphoid tissue, and their outer portions have suffered mechanical removal in places. There is no surface necrosis of the glands or interglandular projecting lymphoid tissue. The hyperplasia of the lymphoid tissues is characterized

[1] For cultures of both these organisms we are indebted to Dr. N. McLeod Harris, of the University of Chicago.

by the presence of large cells scattered throughout the mucosa and submucosa in wide zones about the lymph nodes, the proportion of such cells over the small lymphoid cells of the node increasing with the distance away from the center of the node. The lighter-stained germinal centers of the lymph nodes are small, and cell division is not marked. In the outer parts of the nodes the large cells are first met with, and in such places the usual arrangement of the lymphoid cells in rows is absent. The large cells[1] are two to four times the size of the small lymphocytes both in their nuclei and the cytoplasm. They often contain two, three, or even more nuclei, and in shape alone resemble, in some instances, the polymorphonuclear neutrophiles; their much greater size and the fainter stain of the nuclei, however, are conspicuous differences. Their nuclei stain like those of endothelial cells. Phagocytosis by these cells is not marked in the intestine, inclusions of either red cells or lymphocytes being rarely found, but these large cells are frequently in process of dividing. It is difficult to discern any relation between the large cells as regards origin and the endothelium of either blood or lymph channels. They occur in rows between the fibers of the submucous coat where there are no channels, and in such places mitotic division is abundant. Many of the small veins seem to be packed with these cells. Polymorphonuclear leucocytes and plasma cells are very scarce in these intestinal lesions, and the changes described are found only in connection with the lymphoid tissues of the bowel, the intervening intestine showing no changes. From this description it is evident that the process in the bowel differs from the changes peculiar to typhoid fever as described by Mallory[2] in certain details—its lesser severity, the limitation to the outer zones of the lymph nodes, the relatively little phagocytosis, and absence of ulceration.

In the capillaries of the liver these large cells are very numerous, and in a few small places, scarcely larger than the field of the immersion lens, the liver cells are absent, and these cells are aggregated with a few polymorphonuclear leucocytes. A few of the large cells contain red cells and masses of hemoglobin, and a few are found with dividing nuclei.

Examination of the lymph glands reveals the wide sinuses filled mainly with the large cells, giving an appearance not unlike some forms of tumor metastases, especially the secondary growths in the inguinal glands from the so-called sarcomas of the testicle. The appearance is also somewhat similar to the large-celled hyperplasia of tuberculous lymphadenitis. The cortical nodules and germinal centers are small, and the large cells, as in the intestine, are first met with in the outer zones of the nodules. Inclusions of red cells are more numerous than in the bowel or liver, and phagocytosis of other cells is difficult to find. Dividing nuclei in these cells are very abundant, 12 to 20 in a single immersion-lens field being readily found. In some of the glands hemorrhages have taken place, and in many the sinuses contain a great deal of blood. Partial and total occlusion of sinuses by thrombi occur in some glands; plasma cells are scarce. In the adipose tissue of the mesentery there are large districts filled with these large cells, and dividing nuclei are very numerous among them. Numerous large lymph channels also occur filled with the large cells.

The changes in the spleen are the presence of many of the large cells in the sinuses, with included red cells and dividing nuclei. Masses of hemoglobin, made up apparently of agglutinated red cells, also occur in the sinuses. Serial sections in paraf-

[1] Throughout the description these cells are referred to as "large cells," and a discussion of their nature is taken up later.

[2] *Jour. Exp. Med.*, 1898, 3, p. 611.

fin of entire Malpighian bodies show no changes in these structures. The plasma-cell content of the spleen is not excessive; many are aggregated about masses of blood pigment.

In the adrenal there are foci of necrosis similar in all respects to those in the liver, except that they are more minute. The large cells in the capillaries are found free, with dividing nuclei and inclusions of red cells or hemoglobin masses, occasionally with included mononuclear leucocytes, but the capillaries do not contain the great number of these cells seen in the corresponding channels in the liver. Small aggregations of two to four or six plasma cells and mononuclear leucocytes occur in the capillaries.

In the tunica propria of the esophagus the changes are interesting because limited to a hyperplasia of large cells. The process is easily traced to the perivascular tissues where lymph channels course and have their origin. Just without the most minute arterioles close to the epithelium and in the dense fibrous tissue these cells occur in small groups and short rows. Evidences of their multiplication, mitotic figures, are not numerous in such places, but a little deeper in the wall where the vessels have slightly larger calibers there are found large swollen channels filled with these cells. These channels have walls like veins, but the absence of blood and their content of large cells speak for lymph channels. Between these two places in the esophageal wall, the most minute perivascular groups of these cells, and the large channels filled with them, there are collections without the blood vessels of such cells with numerous dividing nuclei as numerous certainly as in many of the more slowly growing carcinomas. The lymph nodes in the tunica propria of the esophagus where these changes are found are small, normal, and quiescent. There are no signs of phagocytic processes in the esophagus.

In sections of the tongue changes similar to those in the esophagus occur, but the process is not as marked, mitoses not as numerous. Deep in the musculature of the tongue arterioles occur in which most of the cells are the large cells. Other vessels have apparently a normal blood content.

The stomach shows no important changes. The lymph nodes in the mucosa show no signs of cell production; plasma cells are abundant between the glands.

In sections of the lung excellent opportunity is found for study of the finer details of the large cells. Their most striking feature has already been mentioned—a similarity to the polymorphonuclear neutrophiles in the shape of the nuclei. Many have two nuclei, the nuclei are rarely round when single, and often two large hemispherical nuclei lie together by the flat sides; in many the nuclei are apparently clusters of small nuclei. When the nuclei are large there is but little cytoplasm. An excentric position of the nucleus occurs in some cells, but is not common. No granules occur in the cytoplasm. The gigantic nuclei in some of these cells remind one forcibly of certain marrow cells, and especially when the nuclear material is so aggregated that clear portions remain in the center. In some of these cells there are inclusions of either polymorphonuclears or lymphocytes, but inclusions generally, and of red cells in particular, are not numerous. The dense appearance of the chromatin in some cells and their odd shape suggest the results of fixation upon dividing nuclei; in others the chromosomes are quite definite and the phases of mitosis easily recognized. Most of the small arterioles contain these cells abundantly, in those with walls two to four times the width of the endothelial nucleus the content is almost entirely of these large

cells. In the capillary network they are so numerous that the resemblance to leukemia is again striking (see Fig. 2).

In the vessels of the pancreas, especially the capillary network, it is difficult to find the large cells; in the myocardium and thyroid they are present, but not abundantly.

The changes in the kidney are remarkable. In sections stained with hematoxylin and eosin there are seen, with the unaided eye, sharply demarkated, dark, oblong areas in the cortex, the largest 5 mm. in length (see Pl. 4, Fig. 1). As a rule, these are wider near the cortex, but some have a fairly uniform width of 1 mm. or less. Some smaller dark spots occur just beneath the capsule unconnected with the deeper oblong areas, and they are flattened out against the capsule, their long axes parallel to it. In addition to these are smaller dark patches in the cortex, but almost invariably slightly oblong and, like the first mentioned, with their long axes at right angles to the capsule. In the base of the medullary pyramid also are dark patches, but not as dark as those in the cortex and with very vague outlines. With a hand lens or the low power of the microscope these patches are found quite uniformly limited to the medullary rays, except where in the outer parts of the cortex, widening out, they include the labyrinth intervening between the rays. With higher powers these appearances are found to be due to the aggregation in these places of cells mainly of the type described as prevalent in the lymphoid tissues and uterus. At the margin of the infiltrated region the capillaries are dilated and filled with these large cells—a condition that becomes more marked as the regions are entered. It is only by prolonged search that the large cells are found in process of migration through the capillary walls; migration of the small lymphocytes, although not at all a marked feature, is more readily found. The process by which the large cells have reached the outside of the vessels seems to be for the most part through rupture of the distended capillaries. About and within the margins of these cell aggregations there are, between the capillaries and basement membranes of the tubules and parallel to them, long rows of large cells. In the capillaries adjacent to such rows there are also many of the large cells, by actual count as many as, or even more than, of red cells; yet practically none of the large cells are in process of migration through the wall. The cells in the rows between

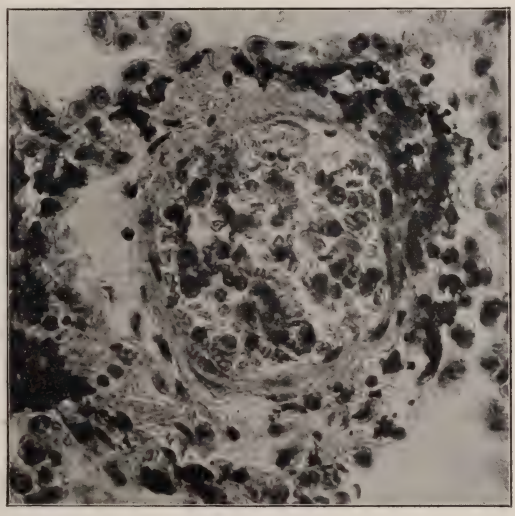


FIG 2.—Showing the great number of large cells—large mononuclear leucocytes—in an arteriole in the lung and the resemblance to the appearances met with in leukemia. The vessel is surrounded for the most part by a deposit of coal pigment. (×375)

capillaries and tubules have evidently attained their location by infiltration outward from the denser portions. The explanation for the accumulation of these large cells in such remarkable numbers by a dilatation and rupture of the capillaries is not in accord with the absence of hemorrhage as a conspicuous feature of the focal lesions. Some free red cells occur within the field of infiltration, but they are not numerous. That greater hemorrhage has not taken place may be accounted for by the blocking of the capillaries with the large cells and by compression of the vessels within the regions; for the large cells without the capillaries have multiplied extensively. The increase through karyokinetic division of the large cells is an important feature explaining the cell masses. In practically any field of the oil immersion lens six or eight dividing nuclei may be found in these areas, and they are almost invariably in cells without the vessels. We have been unable to find dividing nuclei in the endothelium.

The glomeruli are not seriously altered except where they have been included in the aggregations of cells or otherwise secondarily affected. Where the cells are most densely collected the renal parenchyma is absent. Some tubules contain hyalin casts, and in the epithelium of some, especially the collecting tubes of the pyramid, there are round, brown granules. Good fixation was attained, the brush markings of convoluted tubule epithelium being quite generally present. In the base of the medullary pyramids the infiltration with these cells is more uniform, not so dense as in the cortex, and this explains the absence of sharp demarkation for the dark regions seen in such places when examined with the unaided eye. Deeper in the pyramid, however, there are focal lesions, but small, and it is in these minute collections that the method of origin of the lesions is most readily traced to a blocking of the capillaries. These small foci are located always where the parallel vessels, brushlike in their arrangement, are found; they are rarely wider than the width of two or three of the adjacent collecting tubules. In the pelvic mucosa there are aggregations of these large cells and more of the small mononuclear leucocytes than are found in the more proximal lesions, and in such exudates hemorrhage has occurred. Short, thick bacilli, corresponding in size and shape with typhoid bacilli and destaining by Gram's method, are found in the blood vessels, and aggregations of cells in the cortex; in the pryamids they occur in the collecting tubules where there is no evidence of inflammatory processes. They also occur in the blood vessels of the pyramid in the small cell aggregations described, and are numerous in the lesions of the pelvic mucosa. It is impossible to find the bacilli within cells, the large cells especially being examined with that in mind.

The changes in the uterus are entirely in the musculature. At first glance, with the low powers, one is reminded of leukemia, but a moment's inspection determines that the collections of cells are without the blood vessels and in lymph channels. Just beneath the endothelium of the large venous sinuses the cells are met with, and in all respects the appearances are quite similar to the changes in the esophagus. The cells are the same large cells, and a short distance from the endothelium of the large veins the lymph channels are found distended with them; mitotic nuclei are abundant (see Pl. 4, Fig. 2). There is considerable loose fibrous tissue about these large veins, and where the section has been across the veins and their channels are somewhat stellate, from the edge of the projecting rounded fold, marking the lining of the vessel to the musculature, the loose tissue is the seat of a very active production of these cells. There are no lymph nodes, but shortly beneath the vein lining the cells become arranged in rows which terminate in channels with thin walls and no content of red

cells. The lymph channels course parallel with the blood vessels. This change is more marked toward the lining of the uterus than toward the serosa, and in many places in the musculature there are rows of the large cells where lymph channels are not evident nor blood vessels of dimensions larger than capillaries.

The changes in the musculature of the bladder are similar to those in the uterus, but not so marked. Hemorrhages are not limited to the mucous and submucous coats, but are present in the musculature. In places the lining of the bladder is absent. The alterations of the inner coat resemble those in the renal pelvis.

The production of the large cells in the ovary is marked; mitoses are abundant. The lymph channels are not so readily traced as in the uterus; the cells are less commonly in rows. Large regions occur in which these cells are so abundant that with the higher powers the field is quite like the lymph glands.

The cultural reactions of the bacillus isolated entitle it to a place in the group of organisms intermediate between the colon and typhoid bacilli; the transient acidity and terminal alkalinity in litmus milk and neutral red agar refer it to the secondary group which includes the "paratyphoid B" of Schottmüller, de Feyfer and Kayser, *Bacillus enteritidis*, and the organism of hog cholera.[1] Some doubt may remain as to which of these it is most closely related,[2] since interagglutination experiments were carried out with immune serum produced by known strains of paratyphoid organisms, and not with the other members of the intermediate group such as *B. cholerae suis* and *B. enteritidis*. Its widespread occurrence in the body shown by the cultures made at the postmortem examination, its recovery from the blood during life, and the presence of bacilli in the tissues, all indicate its etiologic significance. Bacteria of the hog cholera group may cause sudden acute disease such as occurs in food poisoning, but their etiologic rôle in prolonged illness with some of the features of typhoid does not seem so probable. The relatively high agglutinative powers of the immune serum for known strains of paratyphoid bacilli[3] also to some degree indicate a lack of close affinity with *B. enteritidis*.

Material from the bone marrow was not obtained at the postmortem examination, nor any blood at that time for subsequent agglutination experiments; the reports of failures of the blood serum to agglutinate typhoid bacilli, during the life of the patient, are quite trustworthy; it is quite difficult to account for the changes in the

[1] Boycott, *loc. cit.*

[2] Trautmann (*Zeitschr. f. Hyg.*, 1903, 45, p. 139) prefers to call them all paratyphoid organisms.

[3] See the tables of interagglutination by Boycott.

kidneys by an ascending infection of the urinary tract; a remarkable discrepancy exists between the leucocyte counts made during life and the great numbers of nucleated cells found in the vessels of many of the organs. All of these facts in one way or another affect the relations of the conditions in this case to existing classifications of disease-processes, for this interesting array is presented: symptoms leading to suspicions of typhoid fever; later a purpura hemorrhagica clinically; an anatomic diagnosis of acute interstitial nephritis and typhoid fever, the latter tentative, at the postmortem examination; bacteriologically a generalized infection with a paratyphoid organism; and histologically many of the features of an acute lymphatic leukemia.

In the pathological anatomy of paratyphoid fever as depicted by Wells and Scott,[1] the absence of lesions in Peyer's patches and the solitary follicles is emphasized. In the case since reported by Ascoli,[2] however, an enormous number of freshly formed cicatrices—death on the thirty-third day of the disease—were found in these structures.[3] In both Ascoli's case and the one reported here the mesenteric glands were markedly swollen, a feature only recorded in one of the five cases collected by Wells and Scott. Other exceptions in our case are the proliferative changes in the intestines, the occurrence of the large ("endothelial") cells in the focal necroses in the liver, and no remarkable splenic enlargement. It is difficult to exclude Ascoli's case as one of genuine paratyphoid fever, since the repeated tests of the serum during life were made with a typhoid bacillus of high agglutinability toward known typhoid immune serum, while the bacillus recovered from the spleen during life, and from the spleen and blood postmortem, corresponded in its cultural reactions to the paratyphoid bacillus, variety "B," although its ability to ferment a number of carbohydrates is not recorded.

In the report by Tuttle[4] of an epidemic of paratyphoid fever affecting seven persons, there are many circumstances similar to the conditions related in accounts of food infections, but the opinions expressed in the discussion were in favor of its origin in the drinking-water obtained from a tank. Nothing was discovered, however, when the tank water was examined. The description of the lesions

[1] *Loc. cit.* [2] *Loc. cit.*

[3] Swollen Peyer's patches were the only lesions found in the one fatal case of food poisoning (seven persons) due to the paratyphoid bacillus "B" reported by Vagedes (*loc. cit.*).

[4] *Proc. N. Y. Path. Soc.*, 1903, 3, n. s., p. 185.

found in the postmortem examination of the one fatal case, although not very detailed, confirm the statements of Wells and Scott as to the pathological anatomy. The spleen was not markedly enlarged, $6\frac{3}{4}$ oz., but the intestinal lesions were limited to the lower two inches of the ileum, where there were four or five small ulcers. Both large and small bowel contained large amounts of blood.

The purpuric hemorrhages in this case are quite similar to those observed by Sacquépée[1] in eight soldiers of the garrison in Rennes who within a short time of one another became acutely ill; his account is in many respects like those of food poisonings, but the source of the infection was not determined. In some the skin lesions were roseolae; in others, dark-red patches, disappearing after 8 to 12 days, leaving small, brown-pigmented areas. The serum—1 to 500 to 1 to 2,000—of these patients agglutinated the "B" form of the paratyphoid bacillus, and that organism was recovered from the blood with a coccus (enterococcus Thiercelin) in four cases; in the other four the coccus alone was obtained. Intestinal hemorrhages were a clinical feature of the case reported by Wells and Scott.

There are a number of reasons for excluding an ascending infection of the urinary tract in this case: the active production of the large cells in the lymph glands and intestinal lymphoid tissue, and the evidence afforded by the uterus, bladder, and ovary that in all likelihood the process was even more widespread than demonstrated; the evidence that these cells were carried to the kidney in the blood current; and finally the character of the process in the kidney. The usual ascending infections of the kidney cause an inflammatory process characterized by a peculiar sequestrating necrosis, and the changes are very marked in the collecting tubules of the medullary pyramids. Not only were such changes absent in the kidney of this case, but it is doubtful if the process of infiltration with the large cells and their subsequent multiplication can with propriety be considered as an inflammation. The process in the kidneys in scarlatina and diphtheria designated by Councilman[2] as nephritis (acute interstitial) in one respect at least resembles an inflammatory process more than the lesions in this case; he found that the lymphocytes and their derivatives, the plasma cells, migrated through the vessels; migra-

[1] *Arch. de méd. expér. et d'anat. path.*, 1905, 17, pp. 718–28.

[2] *Jour. Exp. Med.*, 1898, 3, p. 393.

tion of the large cells in this case through the vessels is so inconspicuous and difficult of demonstration that it does not account for the focal collections. Then, too, the large cells do not correspond to the leucocytes (polymorphonuclear, eosinophilous, and small lymphocytes) which, with red cells, constitute to such a large extent the adventitious cells of both acute and chronic inflammations. With the exception of an absence in this case of extraglomerular collections of the large cells, the focal lesions are quite similar in their distribution to those described by Councilman in acute interstitial nephritis, subcapsular, about the pyramidal veins, in the boundary zone and the medullary rays of the cortex. The macroscopic appearance of the kidney also is that of acute interstitial nephritis and totally unlike acute ascending inflammations.

As to the character of the large cells, they resemble mostly the large lymphocytes, and in this conclusion Dr. Joseph A. Capps agrees with us, although he suggested a similarity between some of them and myelocytes. In many respects they correspond to the cells Mallory[1] refers to as "endothelial cells," in their occasional inclusions, the curved, indented nuclei, and marked proliferative capacity. Indubitable origin from pre-existing endothelial cells was not demonstrated for these large cells, although it is probable that the lining cells of lymph channels, at least of the very minute, contributed to their production. In conclusion, the similarity with lymphatic leukemia of the histologic changes and the absence of any notable increase of leucocytes in the circulating blood seem irreconcilable; a similar idea impressed Councilman in his study of acute interstitial nephritis. The necessity of a broader conception of inflammation to include such processes of hyperplasia, metastasis, and proliferation in the secondary deposits has been emphasized by Mallory,[2] and is well illustrated by this case.

EXPLANATION OF PLATE 4.

Fig. 1.—Illustrating the shape and limitation to the cortex of the accumulations of cells. A slight darkening of the boundary zone is shown, as well as minute foci in the medullary pyramid. (× 7½)

Fig. 2.—The perivascular formation of the large cells in the uterus. The obliquely directed opening is a vein; to the left is a small artery and a lymph channel, black and densely packed with the large cells. In the loose tissue between these structures and the musculature the large cells are numerous and karyokinetic nuclei in them very abundant. (× 175)

[1] *Jour. Exp. Med.*, 1898, 3, p. 611. [2] *Ibid.*, 1900–01, 5, p. 1.

FIG. 1.

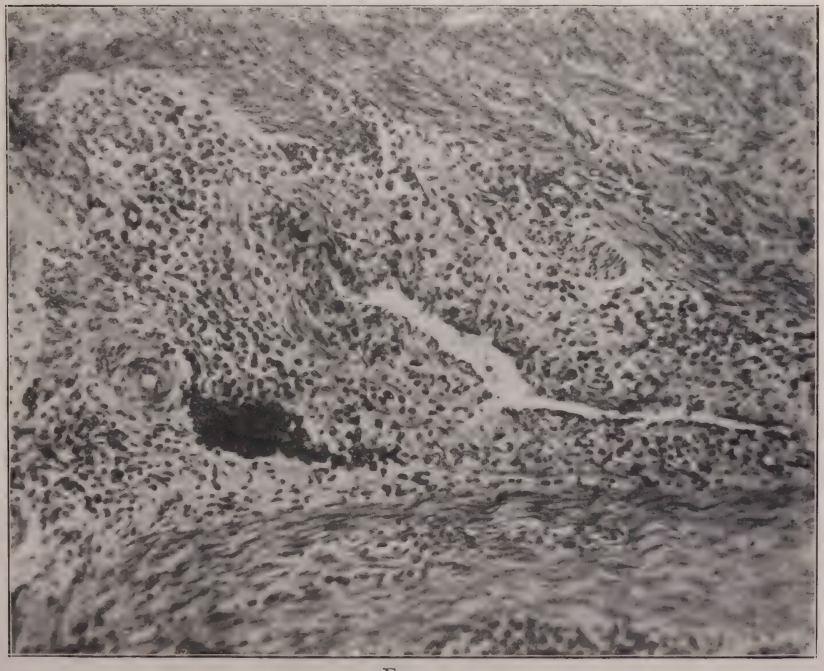


FIG. 2.

SYSTEMIC BLASTOMYCOSIS.

(FINAL REPORT OF THE CASE DESCRIBED BY EISENDRATH AND ORMSBY IN 1905.)

E. R. LeCount and J. Myers.*

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In this article we wish to describe the termination and the results of the anatomical study of the case of systemic blastomycosis reported by Eisendrath and Ormsby[1] in 1905. As stated in their report, the patient was a Polish laborer, age thirty-three, married, and the father of two children. The illness began in February, 1904. The following version of the beginning is given by them:

> The first noticeable departure from his usual good health consisted in a feeling of discomfort involving the chest on the right side, extending through from front to back. This lasted for a time, being better and worse at intervals. In June his first cutaneous lesion appeared. This was located below the left ankle and extended down to the heel, and eventually became a little larger than a dollar. Shortly afterward the other lesions appeared, but it was impossible to tell their exact mode of development. In addition to the area on the left leg, the right leg had several large lesions, also the right and left forearm, and the face, chin, and neck, especially on the right side. About November, 1904, he suffered great muscular weakness, and marked swelling of the feet and ankles occurred. This gradually increased until he was wholly unable to work. He stopped work in December. . . . . On his admission to the hospital in February, 1905, he presented lesions on all the above-mentioned areas, and was very much emaciated, pale, anemic, exceedingly weak, and had some elevation of temperature. Marked edema was present in the ankles, feet, face, and arms. His nails were clubbed, and moderate inguinal adenopathy was noted. . . . . All the lesions were quite superficial, the larger part of each being an ulcer, crust-covered in places, open in others. There was little induration, but considerable sanguinopurulent discharge. The edge of the ulcer was slightly elevated and presented a bluish-red halo, in which there were located a few miliary abscesses. In some parts of the area a papillomatous condition was present. The lesion on the arm was a subcutaneous nodule, which later softened and was incised, and from the sinus left after this procedure, and from both the lesions on the legs, the organism of blastomycosis was demonstrated.
>
> On April 26 blastomycetes were demonstrated in the blood-stained muco-purulent sputum. Tubercle bacilli were not found either in the sputum or in the pus, and there was no reaction to tuberculin. Liver and spleen not palpable May 1. Right apex and upper right lobe dull posteriorly with bronchophony and bronchial breathing. A few râles in right lower lobe. During his four-months' sojourn in the hospital the

* Received for publication February 17, 1907.

[1] *Ill. Med. Jour.*, 1905, 8. p. 454; *Jour. Am. Med. Assoc.*, 1905, 45, p. 1045.

temperature ranged from 98.6° to 102.8°. On July 7, when the patient left the hospital, he was much improved in every way. Five weeks later he suffered a relapse with marked pain in the right chest, several cutaneous lesions were again active, and the sinuses leading from the deep lesions on the forearm had reopened. Eisendrath and Ormsby also report the following cultural and animal experiments:

"The first cultures on March 8 were made from open ulcers and a sinus on the forearm, and although the organism was fairly abundant in smears made from the pus, it failed to grow on the media. On March 22 pus was removed from a subcutaneous abscess on the left forearm, which was inoculated on various media. Six days later growth was plainly visible, and after this time the cultures grew rapidly. These proved to be pure cultures of blastomycetes. . . . . In the pus they occurred as circular and budding forms, having a double contour and the usual refractile capsule. On media the growth varied. It presented a moist, pasty surface on glycerin-agar, with at times a wormy appearance, or else presenting large folds and depressions. Microscopically, these cultures showed many oval and circular organisms, some budding ones, and much mycelial formation, the latter being both coarse and fine and containing sporules. Lateral conidia occurred. On glucose-agar the growth was more dry, white, and presented aerial hyphae; and microscopically there were fewer circular and budding organisms and more fine mycelia. On both glucose and glycerin-agar the media were penetrated to a considerable depth in a semicircular manner. On blood serum the growth was also moist and pasty, and presented a greater proportion of the circular and oval organisms. The cultures taken from the cutaneous lesions, the subcutaneous abscesses, and the sputum all appeared similar. Subcultures had to be made to obtain pure cultures from the sputum. On April 26 two guinea-pigs were inoculated subcutaneously with an emulsion of pus and granulation tissue, removed from the forearm by incision into an unruptured, deep-seated abscess. Both these animals developed a local lesion of blastomycotic infection at the point of inoculation and surrounded it for half an inch in each direction. These lesions remained for a few weeks, then healed, leaving only a slight atrophic area."

Tuberculosis did not develop in any of four guinea-pigs inoculated with pus, sputum, or granulation tissue.

After leaving, as stated by Eisendrath and Ormsby, on July 27, 1905, he returned two months later to the service of Dr. Eisendrath. A gibbus opposite the fourth dorsal vertebra was observed at this time and motion was impaired; the trunk and extremities showed pustular lesions. Sinuses in the forearm and legs were curetted; one of these communicated with the ankle joint of the left side, and denuded bone was demonstrated.

On November 21 he was transferred to the medical service, at first of Dr. Goodkind, subsequently of Drs. Herrick and Wells. His appearance then was that of a fairly well-nourished man who was not very ill. On the arms and feet elevated ulcers were present covered with crusts, also healed ulcers with loose cicatrices. Active ulcers were also present in the right subscapular region; the posterior cervical glands were palpable. The respiratory motion was noticeably lessened on the right side, and tactile fremitus was increased over the right lung behind and laterally, where impaired resonance

bronchial and broncho-vesicular breathing, and numerous moist and whistling râles were obtained. Early in March, 1906, pus began to escape from the gibbus; there are no records of its examination. The hepatic dulness is noted as reaching nearly to the umbilicus in June; new lesions appeared on the face in July, and there were alternate periods of diarrhea and constipation throughout his illness, except toward the end when diarrhea became pronounced. The feces were examined at this time and found to contain considerable quantities of blood and muco-purulent exudate. Dr. Fischmann, who examined them, also reported finding blastomycetes repeatedly with budding forms. Blood also appeared at times in the urine with albumin and casts; a few days before death the urine contained no albumin, but microscopic examination revealed red blood cells and a few casts. Several examinations of the blood were made; a slight leucocytosis was generally found, the different varieties of leucocytes maintaining normal proportions; at no time was an eosinophilia present.

Both knees became ankylosed in a partially flexed position; they, as well as the left elbow joint, were enlarged and tender, but showed no local increase in temperature or redness; extension was difficult and knee reflexes could not be tested. The cutaneous sensibility was not examined. He frequently voided urine with no knowledge of the fact, and during the last four months of his life the bowel movements were also involuntary. The left arm and both legs became edematous; the sinuses mentioned persisted, and on pressure a thick brownish pus of peculiar odor escaped from them. Over both trochanters large decubital necroses developed and a slight general adenopathy. Death occurred during a convulsion on August 23, 1906.

During these months in the hospital an evening rise of temperature usually occurred; the highest was 102.8°. Blastomycetes were repeatedly found in the sputum, and persistent efforts to demonstrate tubercle bacilli gave negative results.

The body was examined a few hours after death and the following anatomical diagnosis made: Blastomycotic bronchopneumonia; blastomycosis of the peribronchial lymph nodes, of the pleura, the subpleural, and retropharyngeal tissue, the liver, the kidneys, the colon, the spinal column (dorsal vertebrae), the external spinal dura, the cerebellum, the left elbow, both knee and ankle joints, and of the skin and subcutaneous tissue with ulcerations, fistulae, and scars. Fibrous induration at root of right lung. Fibrous pleuritis. Passive hyperemia of liver and spleen. Serous atrophy of adipose tissue. Emaciation. Adenoma of thyroid and accessory spleen.

The following are some of the more interesting details: "The body, still warm, is 167 cm. long and much emaciated. There is an abscess over the right parietal bone, edema of both eyelids, a smooth, loose scar on the lower part of the face involving the lower lip; the volar surface of the right forearm has three sinuses leading into as many abscesses, all exuding pus on pressure; pressure on the left elbow, which is twice the normal size, causes flow of pus from several openings in the forearm; this joint and both ankle joints crepitate and fluctuate. About the ankle joints are similar sinuses. There are scars on the legs, and between the shoulders is a large, reddened, edematous, and pigmented region from which many sinuses open; there is a gibbus at the level of the fourth dorsal vertebra.

"The abdominal wall is thin, the muscles pale and atrophied, the peritoneum smooth, the lower margin of the liver 8 cm. below the costal margin in the median line. There are no adhesions in the cavity and only a small amount of fluid. The mesenteric adipose tissue is gelatinous in consistency.

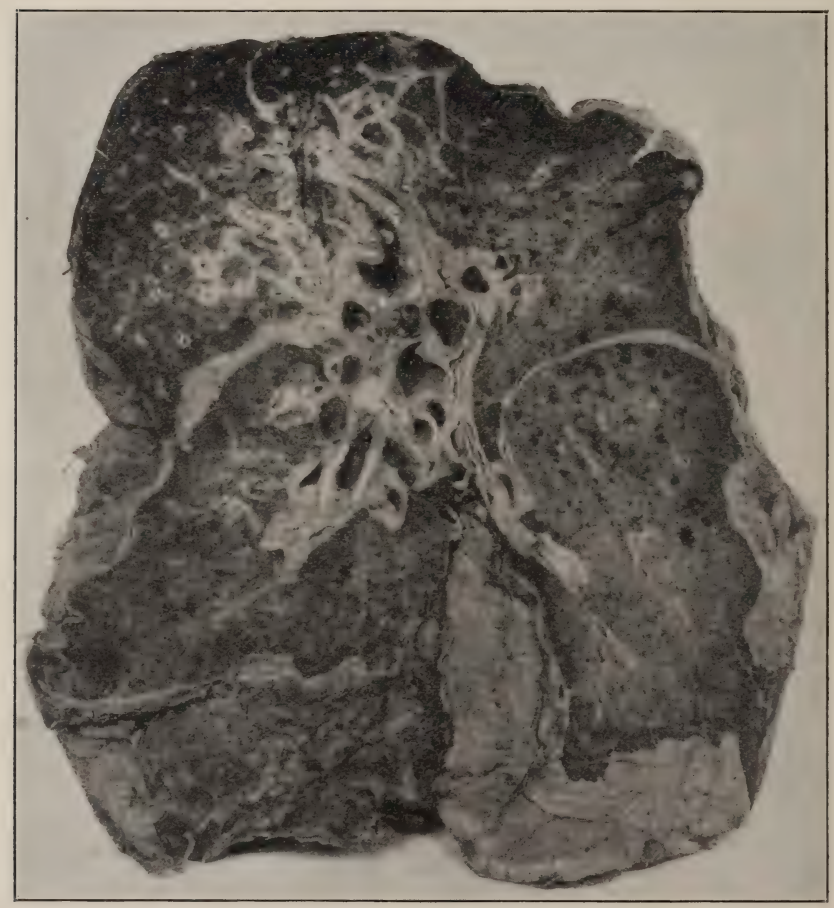

Fig. 1.—The peribronchial fibrous tissue at the root of the right lung, the white zones of infiltration about the smaller bronchi of the upper lobe and the nodules on the diaphragmatic pleura are well shown.

"In the left fifth intercostal space outside the pleura near the angles of the ribs an abscess occurs surrounded by a heavy wall of tissue, the whole as large as a pigeon's egg. In the right pleural cavity are dense adhesions, and between many of them there is a sero-purulent and blood-stained fluid. Separating these adhesions yellowish elevations resembling tubercles are exposed. They vary in size from some just visible, to 2 mm. in diameter; they are very numerous in the pleura over the diaphragm. On and between them are shaggy masses of pink and partially organized fibrin.

"A large amount of fibrous tissue is found on section of the right lung about the vessels and bronchi at the root and in the obliterated interlobar fissures; even near the periphery the bronchi are unusually conspicuous by their thick walls (see Fig. 1). The lining of the large bronchi appears rough; many of medium caliber are plugged with semi-fluid, blood-stained mucus; in the upper lobe the minute bronchi are also closed. The intervening lung tissue shows no changes except marked anthracosis.

"At the root of the right lung there are moderately enlarged lymph glands which show areas resembling caseation; they are otherwise quite black from coal pigment.

"The left lung weighs 300 grams; there are changes similar to those in the right lung in a small region near the apex; the remainder of the lung is emphysematous.

"In removing the lungs and organs of the neck an abscess is exposed posterior to the esophagus and extending upward into the neck from the upper dorsal verte-

FIG. 2.—The large nodule in the cerebellum apparently made up of a number of coalescent smaller nodules with softening in their centers.

brae. Its walls are the two plurae laterally and the eroded vertebrae behind; it has extended upward higher than the erosion of the vertebrae. The pus is thick and yellowish. The anterior surface of the second dorsal vertebra is the seat of a shallow erosion occupying the entire surface; the anterior half of the body of the third, the entire body of the fourth, and posterior half of the body of the fifth dorsal vertebrae are absent. There are found only a few fragments of the fourth, mainly the spinous and articular processes, imbedded in edematous granulation tissue of the abscess marking the gibbus. The intervertebral cartilage between the second and third vertebrae projects forward between the adjacent erosions. The cartilages on either side of the fourth are absent. The changes of the plurae on both sides are in direct continuity with the lesions of the vertebrae, and a part of the upper lobe of the right lung remains adherent. A fistulous passage from the right pleural cavity extends into the spinal canal, opening behind the body of the third dorsal vertebra by an irregular hole 1 cm. in diameter.

'There is a sheath of firm blastomycotic granulation tissue surrounding the

dura opposite the carious vertebrae; this sheath, on the front of the cord, is 5 cm. in length, and here it is 1 cm. higher posteriorly (see Fig. 6). It is very irregular in shape, flattened out in front opposite the destroyed fourth vertebra, and extremely nodular and thick behind at the same point. Cross-section at the greatest diameter shows the width of the tissue outside the dura to be 7 mm. The tissue is made up of easily recognized coalescent nodules, 1 to 2 mm. in diameter, white with darker borders. The spinal fluid below this point is clear.

"Over an area 2.5×1 cm. at the outmost angle of the right lobe of the cerebellum the pia is hyperemic with a pearly white border. This area is smooth, slightly elevated, and the sulci of the cerebellum are absent. A horizontal section here shows that nearly the entire external half of the right lobe of the cerebellum resembles a

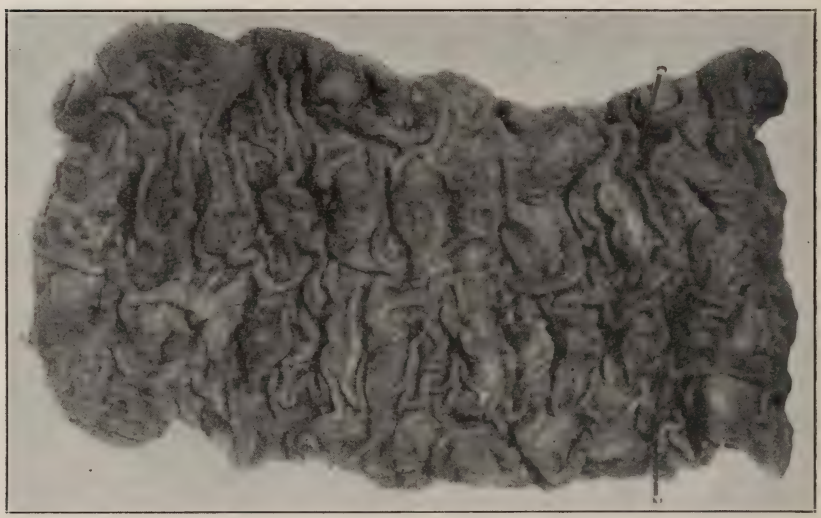

Fig. 3.—The nodules in the colon; careful search failed to reveal blastomycetes.

huge "solitary tubercle" with more liquefaction necrosis than usual in tuberculous brain lesions (see Fig. 2). There are coalescent regions of softening with wide margins of necrotic tissue in which minute nodules, similar to those about the cord, are present. The region so altered measures 4.5 cm. anteroposteriorly and laterally, and 3.8 cm. in its vertical diameter; it extends to within 2 cm. of the median line. The lesion as a whole is deeply seated, only a small portion of its periphery being in contact with the pia; as already described, the process has not extended through the pia. The remaining brain after hardening, and the pons show no gross changes.

"The liver weighs 1,900 grams. There are a few minute areas found on the cut surface resembling tubercles. In one kidney similar lesions occur. The spleen weighs 200 grams and is firm.

"There are many pouches externally on the descending colon arranged in rows between the longitudinal bands; these are nipple-shaped and quite symmetrical both in size and arrangement. The lining is found elevated in folds extending between the pouches and around the circumference of the bowel, and on these folds are conical-

shaped, round, or slightly oblong nodular swellings (see Fig. 3). This condition prevails for a distance of about 15 cm. in the descending colon; the remaining bowel shows no changes. The nodules vary slightly in size and form, but are otherwise very similar in appearance. The largest are from 1 to 1.5 cm. in diameter, have very high margins and a sharply defined central necrosis. In many the central necrotic portion projects higher than the remainder of the nodule. This necrotic tissue is gray, but its junction with the adjacent tissue is the seat of minute hemorrhages."

The relatives desiring as little mutilation as possible, joints and bones were not further examined, nor were attempts made to cultivate blastomycetes, the nature of

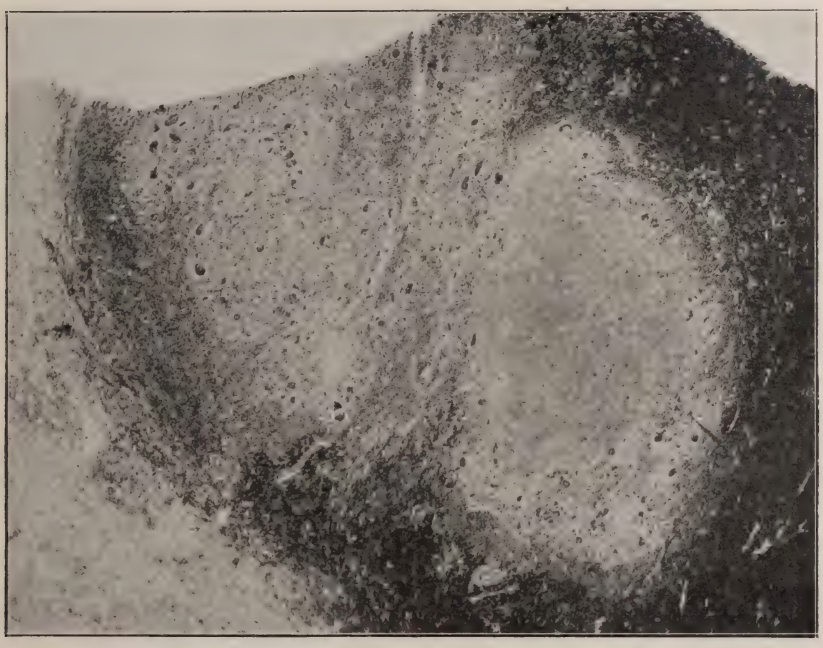

FIG. 4.—Blastomycotic nodules in the bronchial lymph glands. The one to the right shows an eosin-staining necrotic center resembling similar regions in tubercculous lymph glands very closely. (× 25)

the disease having been thoroughly established by the previous observers, Eisendrath and Ormsby.

Cover-glass preparations of the pus in the cerebellar lesion showed an abundance of blastomycetes, and budding forms were easily found. Cultures from the various viscera resulted in the isolation of the colon bacillus only; consequently there was no generalized mixed or secondary bacterial infection as, for example, was found in the case reported by Irons and Graham.[1]

Histologic examination.—Sections of the affected lung tissue show the changes described by Irons and Graham[2] the process is essentially broncho-pneumonic and

[1] *Jour. Infect. Dis.*, 1906, 3, p. 666. [2] *Loc. cit*

more a bronchitis than a pneumonia. In many sections the bronchioles are so obliterated that their location is only evident from the peribronchial deposits of coal dust. The lesions in such places resemble those of tuberculosis very closely. The differences noted by other observers are present; a more highly cellular process, less necrosis, more numerous giant cells with blastomycetes. In adjacent alveoli plasma cells and polymorphonuclear leucocytes are abundant. Farther away from the blastomycotic bronchitis the lung tissue shows evidence of edema with many pigment-carrying cells in the alveoli. The pneumonic process about the large bronchi is peculiar in that the cellular exudate is almost entirely made up of plasma cells. Among these are the

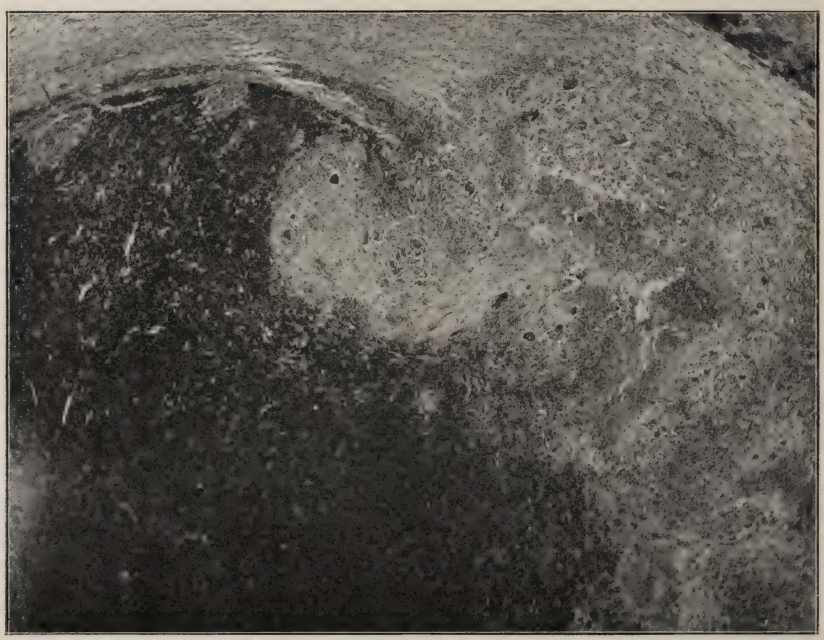

Fig. 5.—Showing the extension outward into the capsule of a bronchial gland. In other places the perilymphadenitis is continuous with the blastomycotic regions in the glands. (× 30)

short rows of cubical epithelium believed to originate by proliferation and metaplasia from alveolar epithelium and common to all chronic inflammations of lung tissue, In many sections the tissue about these large bronchi has more of the characteristics of granulation tissue, and so-called "miliary abscesses," really submiliary and microscopic, occur scattered through it. The centers of these are necrotic, and giant cells with fungi are abundant where the necrotic, tissue joins the surrounding granulation tissue.

In the lymph glands about the large bronchi (see Fig. 4) similar blastomycotic nodules occur; the necrotic centers, as a rule, stain with eosin; some show large amount of nuclear débris. Many of the nodules are continuous through the capsule of the gland with the granulation tissue described about the large bronchi and blood vessels, perilymphadenitis (see Fig. 5). Necrosis in these nodules is not marked, and wide

zones of incircling fibrillated cells with many multinucleated cells constitute, perhaps, the chief feature.

The giant cells almost invariably contain one or more fungi, and many contain coal pigment as well. Parts of the glands are heavily filled with coal dust. The fungi are often budding and in some instances appear to crowd the giant cells; it is very difficult to find blastomycetes except within the giant cells; even small giant cells contain six or eight fungi. The fungi are readily found with ordinary stains because of their characteristic unstained double-bordered rim and the fine granules that they invariably contain. These granules, which are very minute, vary in size slightly and number four to eight in the single organism; they stain with hematoxylin and with basic anilin dyes.

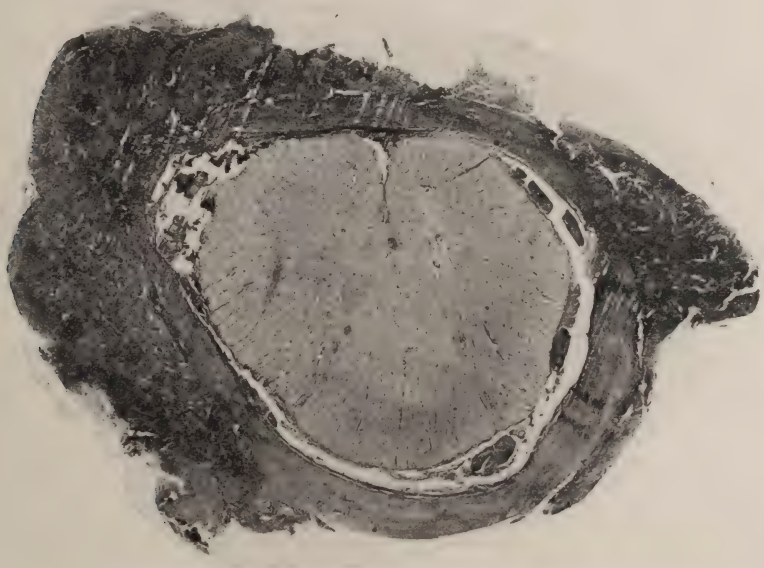

FIG. 6.—The spinal cord and extra dural blastomycotic granulation tissue. The compression and the distortion of the cord is well shown, also the peripheral vacuolation (edema?) in the cord. ($\times 12\frac{2}{3}$)

The nodules on the pleura have the usual structure of blastomycotic lesions. The necrosis in some is extensive. The sections of the liver show no blastomycotic lesions; the liver cells contain quite uniformly small granules of a golden-brown color and appear narrow (atrophy). The capillaries are wide and, in the centers of many lobules, sinus-like. In the spleen there is some hyperemia, but in other respects the organ shows no changes. There is no evidence of amyloid infiltration.

In the colon there is less necrosis than the gross appearances indicated, the chief retrogressive change being the disappearance of the glands in oblong regions as wide as two to four of the glands, due to an infiltration with plasma cells and many eosinophilous leucocytes. In the tunica propria about such regions the lymph channels are filled with similar cells. Many of the glands adjacent to these changes are dilated

and filled with cells in various degrees of necrosis; they appear like minute cysts, but mucin is not present in any abnormal amounts either in them or in the columnar epithelium. Where these changes occur the mucosa and submucosa project toward the lumen of the bowel, and in the latter coat the veins are greatly dilated. The gross appearance of nodular elevations in the bowel no less than the character of the cellular exudate led to the expectation that fungi would be found; although careful search was made in many sections from various places, the results were entirely disappointing; not only were fungi absent, but both giant cells and abscesses as well.

The changes in the kidney, except for minute blastomycotic focal lesions, are not marked. The secretory epithelium is low, and the convoluted tubules have wide channels. There is considerable hyperemia. The blastomycotic lesions are not numerous; many sections show none and some are partially cicatrized without fungi; others possess both giant cells and fungi.

Sections of the stomach, small intestine, adrenals, mesenteric lymph glands, prostate, and testicles show no noteworthy alterations. The changes in the subcutaneous tissues from the region of the gibbus possess one interesting feature. The necrosis is like that in the lymph glands at the root of the lung. In the latter situation the similarity with the necrosis of tuberculosis (see Fig. 4) led to a careful search for tubercle bacilli on the supposition that latent tuberculosis in the glands might be present as well as blastomycosis; no bacilli were found. In other respects the subcutaneous blastomycosis answers to the descriptions repeatedly given in the literature of this subject.

Sections studied from all portions of a thin slice through the largest diameters of the cerebellar lesion show regions of necrosis separated by well-stained tissue. The necrotic tissue, more frequently reacting to hematoxylin than in the lymph glands and lungs, usually forms small areas, but some are 4 to 7 mm. in diameter. Even in the larger eosin-stained necrotic areas the structure can be made out, and it is evident that necrosis was preceded by the changes present in the enveloping bands and partitions of granulation and inflammatory cerebellar tissue. This meshed arrangement is not uniformly definite in the various sections examined, in places the margins are as distinct as is usually the case in either gumma or solitary tubercle. At numerous points, however, this is not the case; two conspicuous features of the process serve to interrupt the sharpness of the margins.

One is the occurrence in the partitions of minute foci corresponding in all respects to the early changes termed "abscesses." Many of these are so minute as to be readily contained in the field of the oil-immersion lens, or even a small portion of it. The smallest consist of a few spaces the size of those in the fat cells; there is some reason to believe these abscesses contained fluid in the fresh condition, probably serum or a few cells resembling lymphocytes. Fungi occur in the most minute abscesses either in the centers or in the peripheries, more often the latter, where they occupy giant-cells which resemble irregular aggregations of cells much more than giant cells of the Langerhans type. In the larger foci of this kind the fungi are invariably in and among the giant-cells which beset the circumference at many points. The abscesses are so abundant in the tissue about the regions of necrosis that the partitions between them in many places appear honey-combed (low powers). The cells in the abscesses are mono- and polymorphonuclear leucocytes, rarely with evidence of nuclear fragmentation or other signs of necrosis, except near the margins of the large regions of necrosis first mentioned.

The other feature of this cerebellar lesion, and one which also constitutes a difference between it and the nodular lesions of both syphilis and tuberculosis of the central nervous system, is the presence in the well-stained partitions, or the tissue incircling the regions of necrosis, of masses of granulation tissue (see Fig. 7). Such masses are not nearly so common as the abscesses described. They are very cellular; approximately one-half are cells with spindle-shaped, faintly stained nuclei, and cells with small round nuclei, apparently of the fixed tissues; the other half is made up of plasma cells, small mononuclear leucocytes, giant-cells, and a few polymorphonuclear leucocytes. There is no order of arrangement in this granulation tissue as in the tissue about the abscesses, nor have the regions any similarity as regards size. Some seem

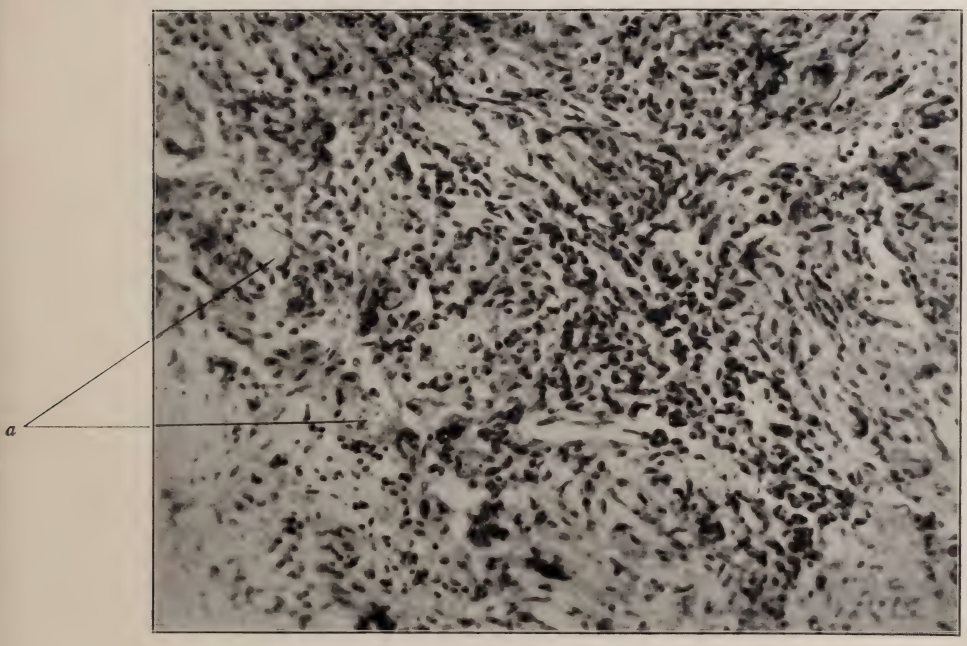

FIG. 7.—The region of blastomycotic tissue in the cerebellum in which the aggregations of young organisms were found and the appearances of sporulation. (*a*) Clusters of fungi. (× 225)

to be formed by the collapse or compression of abscesses. Other larger masses occur in which apparently abscess formation failed to take place or some degree of healing occurred. It is in these larger masses that aggregations of fungi are found remarkable in their great number, the minute size of the organisms, and the absence of budding (see Fig. 8). Budding forms are quite generally met with in blastomycotic tissues; most authors have described them, and in this case they are readily found among the larger organisms in other regions of blastomycosis as well as in that in the cerebellum; but among these clusters of minute forms in this granulation tissue budding is not present to a degree which would account for, or be in conformity with, their grouping in clusters or their number or small size, which last feature, it is believed, indicates

that they are young and newly produced. The hematoxylin-staining granules which the organisms contain do not vary much in size whether the organism is large or small. Many of the blastomycetes in these aggregations are so minute that they are but little larger than the dark granules they contain (see Fig. 7), and the granules are comparable in size with the nucleoli of cells or the granules of chromatin within the nuclei of the plasma cells. With these ideas in mind, search for some other method of reproduction than budding finally resulted in finding large empty organisms, and some of them ruptured, resembling shells or capsules among the clusters of minute young forms. The rounded ends of the membrane at the points of discontinuity are so distinct in some of these (see Fig. 9) that it is difficult to explain the rupture as an artefact.

Fig. 8.—Showing the great number of minute fungi and the absence of budding forms among them, in and about a "giant-cell."

Fig. 9.—Several such empty, large organisms were found with many small forms about them; the rounded ends of the breaks in the membrane of the one represented here were especially distinct.

The idea that sporulation or another method of reproduction than budding has occurred in the cerebellar lesion has still other arguments in its favor, in that the larger organisms contain a number of the dark granules, the minute forms but one, and the intermediate forms are proportionately provided with them (see Fig. 8). Around the granules there is usually a pale unstained region (see Fig. 10), but this does not appear until the organisms have attained some size.

Fig. 10.—The dark granules react to hematoxylin; rarely are more than 8 found in an organism each surrounded with clear zones. The intervening material reacts slightly to hematoxylin.

The extradural sheath of blastomycotic tissue about the spinal cord shows no features of special interest. It is highly vascular, and necrosis is not marked. In places minute foci—"blastomycotic tubercles or abscesses"—occur, but for the most part they are so massed together that their identity is lost or recognized with difficulty. Giant-cells, mainly with peripheral nuclei, are abundant, and the fungi are no more numerous than is usual in blastomycotic granulation tissue; no such clusters of

minute forms as in the cerebellum can be found; as a rule, the giant-cells contain organisms, a few occur free among the cells.

Where the cord has suffered compression (see Fig. 6) and distortion there are found in its peripheral portions many minute holes (edema ?) forming a zone in many sections one-tenth the diameter of the cord; in other sections the entire white substance shows these changes. In numerous places the holes are so large that the peripheral parts of the cord are reduced to a network. In sections stained with the Weigert-Pal method there are degenerative changes in the posterior median columns above the region of compression and marked in the cervical enlargement; at this level there are also similar changes in the direct cerebellar tracks. Opposite the external pachymeningitis marked, almost complete, degeneration was found of one of the posterior roots. Below, there are changes in the pyramidal tracts, more marked in the lumbar enlargement than in the lower dorsal region. These changes are not complete degeneration in any of these locations, except perhaps the posterior nerve root mentioned; they are less marked below than above the point of invasion of the spinal canal; in all places some well-stained myelin sheaths are found when the higher powers of the microscope are used.

One notable feature of this case is the large conglomerate blastomycotic nodule in the cerebellum. In only one other case of systemic blastomycosis, that of Curtis,[1] is there any record of changes in the nervous system, and the statements in that instance are solely clinical, that death was due to meningitis. The reproduction by a process of sporulation demonstrable in the cerebellar lesion is likewise a new feature of the changes encountered in the lesions of this disease. The idea that in the nervous tissues the fungus may have found favorable or different conditions of nutrition, as an explanation for this method of multiplication, is opposed by the facts that the regions in which it was found were very minute, that it was not generally present in the cerebellar process, and that budding was commonly observed in the "abscesses" in the partitions between necrotic regions.

Highly interesting is the relationship between this case of blastomycosis and the coccidioidal disease described by Ophüls.[2] Up to the present two of the chief differences between blastomycosis and coccidioidal granuloma have been the endosporulation observed in the tissues in the latter disease and its tendency to spread by the lymph channels. Although no widespread extension by the lymphatics was demonstrated in the case reported here, the extension to the tracheo-bronchial glands and in peribronchial lymph channels is unmistakable; the endosporulation in the cerebellum in part also

[1] *Ann. de l'Inst. Pasteur*, 1896, 10, p. 448.

[2] *Jour. Exp. Med.*, 1901–5, 6, p. 443; also *Jour. Am. Med. Assoc.*, 1905, 45, p. 1291.

resembles the methods of reproduction described for the organism of coccidioidal granuloma. Taken together, these features in this instance of systemic blastomycosis are in accord with the belief expressed by Ophüls[1] of a close relationship of the organisms in the two diseases.

The failure to find blastomycetes in the nodules in the colon has in no degree impaired our belief in their blastomycotic nature; the gross and minute characteristics of the changes there are so different from any form of ulcerative colitis with which we are familiar, and so much in accord with the lesions of blastomycosis generally, that without the clinical observation of the fungi in the feces it would still seem highly probable that the lesions represent a blastomycotic colitis.

Finally it is apparent that in the growing literature on systemic blastomycosis, new forms of disease, at least anatomically, are constantly being added to those previously recorded. Numerous summaries of the literature have been made; they are of great value in the recognition of new cases; it must be remembered, however, that as yet the carefully studied examples of this disease are few,[2] and that in future observations still other manifestations may be encountered.

[1] *Jour. Exp. Med.*, 1901–5, 6, p. 443.

[2] This is the seventh report of postmortem examination of systemic blastomycosis.

A COMPARATIVE STUDY OF FOUR STRAINS OF ORGANISMS ISOLATED FROM FOUR CASES OF GENERALIZED BLASTOMYCOSIS.*

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THE organisms which form the basis for this study were isolated from the following cases:

No. 1. Disseminated Blastomycosis—Bassoe.[1]

No. 2. Generalized Blastomycosis—Irons and Graham.[2]

No. 3. Generalized Blastomycosis, Case I—Christensen and Hektoen.[3]

No. 4. Generalized Blastomycosis, Case II—Christensen and Hektoen.[3]

For the sake of brevity I shall refer to them throughout this paper as organisms Nos. 1, 2, 3, and 4, respectively. The clinical and pathological details of the several cases are given in the original papers. A second recital would be mere repetition.

This work was undertaken with the hope that progress might be made toward a definite classification in a natural botanical system.

TECHNIQUE.

The usual cultural methods were used—growth on various liquid and solid media at room and incubator temperature under aerobic and anaerobic conditions, etc. In addition, methods employed in botanical research were used, such as the Van Tieghem cell, Wolbach's agar slides, and liquid paraffin cells.

The Van Tieghem cell consists of a hollow cylinder of glass a centimeter in height and diameter. The edges may be ground to secure firm union with glass slide and cover-slip which complete the cell. The glass ring and cylinder are cemented to a slide, and liquid media or distilled water are filled in to about one-third the height of the cylinder. A cover-slip is prepared as in a hanging-drop preparation and inverted over the upright cylinder. It may be fixed with paraffin or balsam. The purpose of the preparation is to afford means of watching day by day, for a period varying at the will of the investigator, the growth and development of an individual cell or group of cells. If the hanging-drop be broth, media of the same density should cover the bottom of the cell. If it be agar, distilled water should be used. By diffusion the inoculated portion remains constant in concentration, evaporation and absorption going on equally.

* Received for publication December 10, 1906.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 91.

[2] *Ibid.*, p. 666.

[3] *Jour. Amer. Med. Assoc.*, 1906, 47, p. 247

Wolbach's[1] agar slides are made by flowing sterilized microscopic slides with melted agar, allowing the agar to harden, and inoculating by streaks with the platinum loop. A cover-slip is then placed over the inoculated surface, and the edges are sealed with a hot wire. I found it more serviceable to seal the edges with paraffin, particularly when the preparation is to last a week or so. The slides and cover-slips may be conveniently sterilized in the hot-air oven by placing them in Petri dishes.

Paraffin chambers were employed for watching cells submerged in distilled water, physiological salt solution, etc., and for anaerobic growths in nutritive liquid media. They are made by painting squares slightly smaller than the cover-slip on sterile slides with liquid paraffin. After hardening, a drop of the solution or medium is placed in the center of the square, inoculated, a sterile cover-slip dropped on, and its edges paraffined down. The preparation is useful in the same way as the Van Tieghem and Wolbach cells, enabling the observer to watch individual cells in liquid environment with a magnification as high as 1,000.

Dehydration with gypsum blocks was not attempted because of the negative results of Busse[2] and Ricketts.[3] A number of inoculations of white mice were made to assure continued pathogenicity.

COMPARATIVE STUDY OF THE ORGANISMS IN CULTURES.[4]

GROSS CULTURAL APPEARANCES.[5]

A. Solid media.

1. Glucose-agar stabs.

a) At room temperature.

No. 1. Fine white, delicate, aerial and submerged hyphae, apparent in 48 hours. Growth increases rapidly so as completely to cover the surface with a white, downy layer and to outline triangular area (base uppermost) along stab, in 7 to 14 days.

No. 2. Same. Firmly adherent to substratum.

No. 3. Same. Aerial hyphae not so abundant. Surface growth consists of waxy, crater-like elevation.

No. 4. Same. Aerial hyphae more prominent. Hyphae coarse and long.

b) At incubator temperature.

No. 1. Raised, waxy, irregularly spherical, yellowish-white, single colony in 3 to 5 days. No aerial or submerged hyphae visible at any time (30 days).

No. 2. Same. Few coarse, short, aerial and submerged hyphae at fifth day. Hyphae increase slowly, together with enlargement of waxy mass. "Porcupine" growth.

No. 3. Same as No. 1. On thirty-first day hyphae were noted. Were not present on seventeenth day.

No. 4. Same as No. 1. No hyphae at any time.

[1] *Jour. Med. Res.*, 1904, 8, p. 53.

[2] *Virchow's Arch.*, 1896, 144, p. 360.

[3] *Jour. Med. Res.*, 1901, 6, p. 293.

[4] Two complete sets of cultures were made with an interval of one month to note changes, should any occur, because of growth on artificial media. The data here given are a combination of both cultures as no marked variations were noticed.

[5] Duplicate inoculations on all media were made simultaneously, one tube placed in the room (temperature varying between 16° and 24° C.); the other in the incubator at 37° C.

2. Glucose-agar slants.
   a) At room temperature.
      No. 1. Elevated, moist, white, pin-point colonies along the streak at the end of 24 hours. Abundant submerged hyphae at end of fourth day. A few delicate aerial hyphae at end of seventh day; abundant submerged and aerial, such flattened and applied to surface at end of two weeks.
      No. 2. Long, coarse, aerial hyphae and abundant submerged at end of fifth day. In two weeks abundant, white, fine, downy, aerial growth covering agar and extending along glass.
      Nos. 3 and 4. Same as No. 1. No aerial hyphae at any time. Pin-head, nodular colonies on surface.
   b) At incubator temperature.
      No. 1. Delicate, elevated, moist, white, pin-point colonies along streak at end of 24 hours. Increase in density at end of two weeks; surface now covered with white, pasty growth, with some evidence of spherical colony formation. No aerial or submerged hyphae at any time.
      No. 2. Same as No. 1. Colonies more discrete, raised and firmly adherent to substratum.
      Nos. 3 and 4. Same as No. 1. Submerged, short, compact hyphae present on fifth day.
3. Glycerin-agar slants.
   a) At room temperature.
      No. 1. Beginning hyphal growth noticed on third day; submerged prominent on fifth day, both increase uneventfully till thirtieth day (last observation).
      No. 2. Same as No. 1. Growth apparent on second day. Aerial hyphae, coarse and vigorous; submerged, fine, and delicate.
      No. 3. Same as No. 1, except that no aerial hyphae are produced at any time (30 days). On surface white, waxy, discrete colonies.
      No. 4. Same as No. 3. No aerial; abundant submerged hyphae.
   b) At incubator temperature.
      No. 1. Elevated, waxy, nodulated growth on fifth day. Submerged hyphae on seventh day, slight. No aerial hyphae at any time. Growth increases slowly; on seventeenth day waxy growth elevated to 3 mm. above surface.
      Nos. 2, 3, and 4. Same as No. 1. Waxy, wormlike mass; crater-like in places.
4. Blood (ox) serum slants.
   a) At room temperature.
      No. 1. Flat, slightly elevated, spherical, pin-head growths. Few coarse aerial hyphae at end of first week.
      No. 2. Same as No. 1. One peculiar, long, coarse aerial hypha 8 mm. long extending from media to glass.
      Nos. 3 and 4. Same as No. 1. Colonies yellow-white, nodulated, more prominent. Apparent on second day.
   b) At incubator temperature.
      No. 1. Delicate, elevated, white, pin-point colonies along streak at end of

24 hours. Growth increases rapidly to form streak of raised, white, starchlike colonies.

No. 2. Same as No. 1. Colonies more spherical and discrete. Two coarse, short, aerial hyphae noted on fourteenth day.

Nos. 3 and 4. Same as on No. 1.

5. Gelatin stabs.

a) At room temperature.

No. 1. Fine, white, delicate, aerial and submerged hyphae apparent in 48 hours. Growth increases completely to cover surface with characteristic, white, downy mass. No liquefaction.

Nos. 2, 3, and 4. Same as No. 1.

6. Potato.

a) At room temperature.

No. 1. Fine, white, delicate hyphae beginning on the third day. Increases to cover entire streak at end of 14 days. Nodular colonies increase simultaneously.

Nos. 2, 3, and 4. Same as No. 1.

b) At incubator temperature.

No. 1. White and brownish-white, raised, compact, pin-point to pin-head nodules apparent on fifth day along streak. Increase until, as potato dries up, growth becomes stationary, white and pastelike.

No. 2. Difficult to get growth at end of two weeks. Few, dried, scattered, pastelike patches on surface.

No. 3. Same as No. 1; growth on third day.

No. 4. Same as No. 1; growth not vigorous.

B. Liquid media.

1. Broth.

a) At room temperature.

No. 1. Fluffy, compact, thistle-down growth beginning in 24 hours. Supernatant fluid clear. Growth increases in size and density. Always strongly coherent.

Nos. 2, 3, and 4. Same as No. 1.

b) At incubator temperature.

No. 1. Fine granular detritus and sediment at bottom of tube in 24 hours. Can be shaken up to form cloud. Some evidence of thistle-down growth on seventh day. Same increases slowly till on thirtieth day thistle-down growth is more strongly in evidence.

No. 2. Short, coarse, compact hyphae at end of 24 hours, increasing slowly and inclosing nodular colonies in mycelial mesh-work. On thirtieth day mycelium increased to resemble growth at room temperature.

No. 3. Fine granular detritus. Slight fluffiness later.

No. 4. Same as No. 3.

2. Dunham's medium.

Indol produced in no instance.

3. Litmus milk.

a) At room temperature.

No. 1. Slight acidity at end of second day. Continues without change for two weeks. No coagulation.

No. 2. No change from control at end of first week. Some time between that date and the end of the second week complete coagulation occurred with slight alkalinity.

No. 3. Slight acidity on fifth day. No coagulation.

No. 4. Possibly slightly acid on fifth day. Later (fourteenth day) no acidity. No coagulation.

b) At incubator temperature.

No. 1. Slight acidity on fifth day. At fourteenth day color somewhat fainter, lighter, and pinker than control. Fat globules on surface.

Nos. 2, 3, and 4. Same as No. 1.

SUMMARY OF GROSS CULTURAL APPEARANCES.

1. The four strains of organisms appear nearly identical, so far as growth in test-tubes goes. A few minor differences are summed up below, under 6.

2. The organisms grow vigorously on the usual laboratory media, with perhaps a slightly more abundant growth on faintly acid glucose-agar.

3. Temperature is perhaps the most important factor in varying the gross and microscopic morphology: room temperature favors production of mycelia and aerial hyphae; incubator temperature inhibits production of hyphae and favors coherent, waxy, yeastlike colonies (budding forms).

4. Those cultures which produce yeastlike growths at incubator temperature develop hyphae within 24 hours when withdrawn and placed at room temperature. Likewise the majority of yeastlike colonies will finally (in 17 to 30 days) show evidence of beginning hypha formation even if kept at 37° C.

5. Glucose-agar stabs and broth form the most serviceable culture media if a limited variety is at hand. Duplicates should always be made to control differences in morphology at room and incubator temperature.

6. The minor differences referred to in 1 are:

a) Firm adherence to substratum of all cultures on solid media of organism No. 2. Growths in incubator of organisms Nos. 1, 3, and 4 are readily removed.

b) Production of hyphae in all cultures of No. 2. It was impossible to obtain pure budding forms. Organisms Nos. 1, 3, and 4 may be obtained in pure budding forms in stab growths in incubator.

c) Formation in the case of organism No. 2 of compact mycelial and nodular growths in broth in incubator, while organisms Nos. 1, 3, and 4 form granular detritus (early) with no mycelium.

d) Coagulation of milk with slight alkalinization by organism No. 2, whereas organisms Nos. 1, 3, and 4 do not coagulate and cause but slight acidity.

MICROSCOPIC APPEARANCES OF ORGANISMS UNDER CHANGING EXPERIMENTAL CONDITIONS.

Observations were made of particular individual cells and recorded from time to time preliminary to studying the methods of hyphal production. Budding forms were inoculated anaerobically[1] on liquid broth and glucose-agar, using the paraffin and Wolbach preparations as described under "Technique." Slides were placed in the room on the warm (37° C.) stage. Budding and hyphal production occurred irrespective of temperature, though the former was more marked on the warm-stage growths.

It was possible to observe the actual process of growth in a few instances. In those cells which were about to bud or sprout the heavy, refractile outer wall was seen to put forth at one point two short, collar-like projections.[2] This undoubtedly was due to a break in the capsule and a drawing-back of the protoplasm on each side. Gilchrist noted and drew these collar-like projections in tissue, but, so far as I know, they have not been seen in cultures before.

The projection of the protoplasm occurs fairly rapidly, resembling closely the projection of pseudopodia by protozoa. The protoplasm which precedes is homogeneous, the finer and coarser refractile bodies remaining in the mother-cell for some time. The mother- and daughter-cells may easily be differentiated for a long time because of the heavy-contoured periphery of the parent cell.

I could detect no difference in the reproductive process which would suggest why in one case buds formed and in another hyphae developed. In some instances both buds and hyphae were seen to arise from the same mother-cell. In any particular field watched for 24 hours all the cells did not show evidences of reproductive processes. In one case four cells out of a field of 27 showed beginning hyphal buds at the end of 24 hours; in another, four out of 10 showed reproductive changes; in a third, three out of 15, etc. It is interesting to note in this connection that the warm-stage slides showed a higher proportion of growing cells than preparations kept at room temperature.

One constant finding on solid media was the production of bodies which may be one of a number of botanical possibilities. I refer to the formation of clusters of botryoidal bodies at the end of hyphae, at the end of short lateral buds, and, in some cases, within a short distance of a sphere to which they are attached with a long, hypha-like stalk. Figs. 1, 2, and 3 show various types of these clusters. In older cultures growing at room temperature in which these bodies were not present, they

[1] The preparations were at least practically anaerobic. The only oxygen which may have been used must have come from that dissolved in the media. No visible "bubbles" of air were allowed in the preparations.

[2] These projections are doubtless the sides of a cylindrical or funnel-shaped collar through which the protoplasm is extruded. The appearance noted is due to refraction in optical section

could be made to form by placing the culture in the incubator (Fig. 3). Forms similar to the one drawn in Fig. 4 were found in younger growths. Apparently the bulbous enlargement at the tip bursts to form atypical clusters. No such actual bursting was ever observed.

The question at once presents itself: What are these bodies? At the present time one cannot decide positively. In no case was it possible to cause the liberated bodies to bud or produce hyphae, although transfers were made into solid and liquid media under aerobic conditions, at room and incubator temperature, etc. For the present, therefore, I believe it would be in the interest of conservatism to regard them as bulbous hyphae with liberated protoplasmic and food granules produced by the bursting of a turgid protoplasmic mass at 37° C. and in an anaerobic, solid medium.

Hyphae, on solid media, grew apparently in the direction of the oxygen supply, as shown by the prevalence of the growth in the proximity of the paraffin border. In those cultures which had been growing for two weeks unusual forms were produced at the distal end of hyphae. Fig. 5 shows the end of such a hypha. Fig. 6 shows a more advanced stage of the same process. It is not clear just what method of growth is represented. It may be simply a vegetative production of lateral branches or a reproductive growth from spores germinating within a mother-cell. In the absence of more exact botanical proof, I should be inclined to regard the former as the real condition present.

The phenomena noted thus far have been those observed for the most part on solid media. Liquid preparations in paraffin cells were also productive of results. Notably the relationship of budding forms to the oxygen supply was well shown in the case of an eight-day preparation of organism No. 3 growing at room temperature in broth. Through the accidental chipping-off of a small particle of the paraffin border, air was allowed to get into the preparation, with a resulting gradual evaporation of the medium. Fig. 7 was made of this slide, *c* to *b* being the part of the slide from which the broth was evaporated; *a* to *b*, the remaining medium and cells. The amount of hyphal production is seen to be dependent on the proportion of dissolved oxygen and proximity to incoming air.

Various experiments of submersion under distilled water and salt solution, etc., were not productive of anything new. In some instances the organisms were killed without change; in others plasmolysis occurred; in others the peripheral theca was seen to be disrupted, liberating contained bodies; in others large chlamydospores (resting cells) were formed (Figs. 8, 9, and 10).

The results of observations of the growing organism may be grouped as follows:

1. It is possible, by appropriate methods, to watch the germination of fungi from hour to hour and day to day for a period of a month or longer. Only by such methods can one hope to arrive at the complete life-cycle.

2. The collar-like projections, observed by Gilchrist in tissue, indicative of a rupture of the capsule, may be observed in artificial media.

3. A temperature of 37° C. favors production of budding forms

(as in gross aerobic cultures) as well as stimulating both reproductive and vegetative germination.

4. Involution forms are produced in from three to eight days or longer in anaerobic glucose-agar cultures. These should be regarded, for the present, as burst hyphal enlargements. It cannot be said that the enlarged, ramified enlargements of older (two weeks) hyphae are asci.

5. The organisms are facultative anaerobes on glucose-agar, but obligate aerobes in broth.

6. Immersion in distilled water, KOH, etc., resulted in death of cells, with or without plasmolysis, rupture of cells in some cases, formation of large resting cells in others.

FERMENTATION EXPERIMENTS.

Eight sugars were used as a basis for these experiments: dextrose, lactose, levulose, nutrose, galactose, maltose, mannit, saccharose; and also *inulin*. Fermentation tubes were inoculated with the four organisms (nine each) and placed in the incubator for 16 days. In no case was carbon dioxide generated. A few of the tubes showed gas bubbles at the top. These were submitted to the KOH absorption test for CO_2. Alcohol and acetic acid were not tested for. In view of the negative results, we must conclude that the four organisms are not sugar-inverters and fermenters and may be classed with similar organisms in Ricketts' group, of which the fungus from Case 12 may be taken as a type.

ANIMAL EXPERIMENTS.

White mice were used as hosts. Because of lack of time a few only were injected, and these simply to assure pathogenicity. The injections, consisting of a 0.5 to 0.8 c.c. of a salt suspension of budding forms, obtained from agar colonies growing at 37° C., were made intraperitoneally with the usual precautions. All of the mice died in from four to 11 days after the injection. Typical subcutaneous abscesses formed in the cases of mice inoculated with organisms Nos. 1 and 4, and characteristic budding forms were found in the pus. Organisms were not recovered from the other mice.

SUMMARY AND CONCLUSIONS.

1. Four strains of organisms isolated from four cases of generalized blastomycosis appear identical.

2. Pronounced variations in the gross and microscopic morphology of the organisms are produced by variations in temperature. As a routine for purposes of study cultures should be grown at both room and incubator temperatures.

3. Botanical methods for watching the development of individual cells are the most useful for studying the life-processes of fungi. By

PLATE 5.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

PLATE 6.

FIG. 5

FIG. 6.

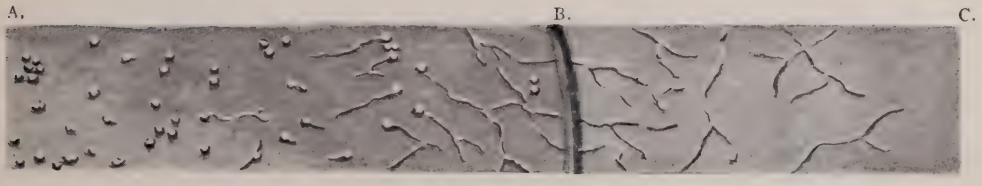

FIG. 7.

FIG. 8.

FIG. 9.

FIG. 10.

means of such methods many unusual morphologic forms may be observed.

EXPLANATION OF PLATES 5 AND 6.

Fig. 1.—Typical ruptured hypha (sporangium (?) ascus (?)) showing free clusters (spores(?), protoplasmic granules (?)). Organism No. 1; 72-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

Fig. 2.—Another frequent type of ruptured hypha occurring at short distance from vegetating cell. Organism No. 3; 72-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

Fig. 3.—Ruptured hypha occurring in oidial type of growth. This culture at the end of seven days' growth at room temperature had not produced typical group clusters. Accordingly the slide was put into the incubator, whereupon, in 24 hours, the phenomenon occurred. The more lightly shaded, less refracting body with attached clusters was produced during that time. Organism No. 3; 11-day anaerobic growth on slide. ×600.

Fig. 4.—Enlarged bulbous extremity of hypha before rupture. Shows manner of production of group clusters. Organism No. 1; 67-hour anaerobic growth at 37° C. on glucose-agar slide. ×1000.

Fig. 5.—Tip of old (15 days) hypha showing unusual (involution(?)) morphology. Asci (?); a lateral branch formation (?). Organism No. 2; 15-day anaerobic growth at 37° C. on glucose-agar slide. ×1000.

Fig. 6.—Same as Fig. 5. Later period of growth. Organism No. 2; 15-day anaerobic growth. ×1000.

Fig. 7.—Hyphae production in liquid medium and its relation to oxygen supply. No hyphae were produced until, through the accidental chipping-off of the paraffin border, air was allowed to enter, *b* to *c* being the part of the slide from which the broth has evaporated, *a* to *b* the remaining medium and cells. The hyphal production may be seen to be dependent on the proportion of dissolved oxygen and proximity to incoming air. Organism No. 3; eight-day growth at room temperature in parraffin slide of broth. ×80.

Fig. 8.—Chlamydospore (resting cell) occurring after 72 hours' submersion in distilled water. Organism No. 3; 72-hour culture at room temperature in distilled water. ×1000.

Fig. 9.—Plasmolysis occurring after four to eight days' immersion in 0.85 per cent NaCl. Organism No. 4; eight-day culture at 37° C. in physiological salt solution. ×1000.

Fig. 10.—Ruptured sphere and extrusion of cell contents after mounting culture in 1 per cent KOH solution. Organism No. 1. Slide prepared after 72 hours' growth on aerobic glucose-agar slant. ×1000.

DIPHTHERIA: A STATISTICAL STUDY OF CERTAIN LABORATORY AND CLINICAL OBSERVATIONS.*

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VITAL statistics relative to diphtheria have been collected from time to time and have proved of great importance. Statistics relative to laboratory findings and their relation to the clinical aspects of the disease have, however, not been so systematically collected and studied, and there are comparatively few tables of much value. Those which are accessible refer almost entirely to urban conditions, very few to rural communities.

The bacteriological laboratory of the Iowa State Board of Health has now been in existence a little more than two years, and during that time has made 6,155 examinations (February 16, 1907) for the presence of diphtheria bacilli for the physicians of the state. These examinations were made both for diagnosis and for release from quarantine. The conditions upon which release from quarantine is based in Iowa are as follows:

> Quarantine shall be released in those houses in which diphtheria has been diagnosed when synchronous cultures taken from the noses and throats of all infected persons quarantined show two consecutive negative examinations, providing the regulations of the board regarding disinfection and fumigation have first been complied with.
>
> Those who have been quarantined with diphtheria patients may be released from quarantine where both nose and throat cultures on examination by the bacteriologist of the State Board of Health do not show the presence of diphtheria bacilli.
>
> In districts where it is not possible or desirable to use the laboratory findings as a means of regulating quarantine, those suffering from diphtheria shall be quarantined for a period of not less than four weeks from initial symptoms where antitoxin is used, and five weeks where antitoxin is not used.

In order that the statistics to which I am about to refer in detail may be properly understood, it should be stated that diagnosis outfits containing culture media are kept in every city and town in Iowa in places called culture or supply stations, from which they may be obtained at any time by physicians. These diagnosis outfits contain

* Received for publication February 20, 1907.

a card for the collection of statistical data, on which is printed the following:

FRONT.

DIPHTHERIA.

[To be filled out by physician only.]

[See also other side.]

Patient's name.....
Address.....
Physician's name.....
Address.....
Health officer's name.....
Address.....

[To be filled out at laboratory only.]

Case No.....Report, mail—telegraph
Diagnosis .....
Received .....
Reported .....
Examined by.....
Notes .....
.....

Shall report be sent by mail or telegraph?.....(Telegraphic reports are at physician's expense.)
Date of culture—Year.....Month.....Day of month.....Hour.....A.M.....P. M.
No. of culture from the patient, whether 1st, 2d, 3d, etc.....For Diagnosis or Release.....
Culture from nose or throat or both.....(release culture must be from both nose and throat.)
Age.....Sex.....Occupation.....Married or single.....
How long since disease commenced?.....What is suspected source of infection?.....
If child, what school has he or she been attending?.....
How many other cases of diphtheria (if any) in the house?.....

(OVER)

BACK.

[To be filled out by the physician only; see other side also.]

Membrane present.....Nares.....Pharynx.....Tonsils.....Larynx.....
Exudate present.....Nares.....Pharynx.....Tonsils.....Larynx.....
If no membrane or exudate exists, state site of inflammation.....
How long since an antiseptic was used in the throat.....Pulse.....
Temperature.....Respiration.....
Constitutional symptoms.....
Has antitoxin been used? How much and when?.....
Clinical diagnosis.....
Remarks.....
.....

(Please fill out both sides of card as indicated for every preparation made)

IOWA STATE BOARD OF HEALTH, BACTERIOLOGICAL LABORATORY,
(UNIVERSITY OF IOWA) IOWA CITY, IOWA

(OVER)

After the bacteriological examination has been made, the report is sent on a form like that given below:

FRONT.

It is recommended that no culture for release from quarantine be taken prior to fourteen days from date of the development of the disease.

A second culture for release from quarantine should not be taken until report of first culture is received.

IOWA STATE BOARD OF HEALTH, BACTERIOLOGICAL LABORATORY,
(UNIVERSITY OF IOWA) IOWA CITY, IOWA

IOWA CITY, IOWA,.....

DR.....
....., Iowa.

DEAR DOCTOR:

The culture No.....form.....
submitted by you has been examined.
Diagnosis: .....(see other side)
Special remarks.....
.....
.....Bacteriologist.
.....M.D.
Director.

(Every case of diphtheria must be reported to the local Board of Health.)

(OVER)

BACK.

INTERPRETATION OF DIAGNOSIS GIVEN.

1. DIPHTHERIA BACILLI PRESENT, indicates that—

a) The patient has diphtheria or has recently had it; or

b) The diphtheria bacilli are present, even though no lesions of the disease exist (as occasionally occurs, especially among nurses). Such individuals are capable of spreading the disease.

2. NO DIPHTHERIA BACILLI FOUND indicates—

a) The entire absence of diphtheria bacilli; or

b) That the culture was not properly made, due to improper technique either in applying swab to the throat or in smearing the swab over the surface of the medium (please carefully follow the instructions); or

c) That some antiseptic was applied to the throat shortly before taking the specimen. It interferes with the subsequent development of the bacteria; or

d) That the infection was in the larynx, and the swab may not have touched the membrane; or

e) That it is one of the rare cases where only a very few diphtheria bacilli are present at the beginning, and may have escaped observation when making the examination. More will be present in a later culture.

If there is any doubt about the case, send us another specimen.

3. QUESTIONABLE may mean—

a) That *no growth* developed on the media; or

b) That the growth was so *scanty* that no diagnosis could be made; or

c) That the culture media were not in proper condition—too dry or contaminated. Such media should not be used.

When the specimen is labeled "questionable," it will again be examined the following day. If diphtheria bacilli are found, you will be notified at once; if it is negative, no report will be sent. But do not wait—send another specimen immediately, if you deem advisable.

(OVER)

The following records are based upon reports of examinations made as indicated above. Of these examinations, 2,867 were for diagnosis (Tables 1–6) and 3,063 for release from quarantine (Table 7). Only one culture was submitted for diagnoiss from 2,743 patients, two cultures from 124 patients; 1,201 of these were males, 1,412 females; 438 of them were married, 1,639 unmarried, 7 were widowed. In regard to age, 564 were from one to five years, 1,196 from six to 15 years, 761 from 16 to 60 years, nine over 60 years.

TABLE I.

DIAGNOSIS BY PHYSICIAN AND LABORATORY FINDINGS.

A. Diphtheria (by physician):	
Laboratory found diphtheria bacilli	670
Laboratory did not find diphtheria bacilli	437
B. Not diphtheria (by physician):	
Laboratory found diphtheria bacilli	137
Laboratory did not find diphtheria bacilli	734
C. Possibly diphtheria (by physician):	
Laboratory found diphtheria bacilli	151
Laboratory did not find diphtheria bacilli	259
D. Uncertainty (by physician)	
Laboratory found diphtheria bacilli	67
Laboratory did not find diphtheria bacilli	103

TABLE 2.
OCCUPATION.

Agents	4
Bankers	2
Barbers	5
Business (indoors)	138
Clergymen	3
Dentists	3
Druggists	4
Housekeepers	364
Laborers (outside)	135
Lawyers	5
Mail carriers	3
Nurses	14
Physicians	8
Railway employees	19
Pupils and students	1,203
Teachers	27
Veterinary surgeons	1

TABLE 3.
LENGTH OF TIME AFTER BEGINNING OF DISEASE THAT CULTURE WAS SENT.

1 day	648
2 days	516
3 "	403
4 "	212
5 "	128
6 "	62
7 "	119
8 "	18
9 "	13
10 days and over	114

TABLE 4.
SOURCE OF INFECTION.

Not known	1,453
School	395
Family	185
Physician	3
Nurse	2

TABLE 5.
NUMBER OF OTHER CASES OF DIPHTHERIA IN THE HOUSE.

None	1,636
1	192
2	85
3	25
4	22

TABLE 6.
LOCAL CHANGES.

A. Membrane (fibrinous exudate) present:	
Nares	111
Pharynx	598
Tonsils	1,547
Larynx	126
B. Exudate (serous) present:	
Nares	118
Pharynx	784
Tonsils	1,067
Larynx	107
C. No membrane, no exudate, but hyperemia	190
D. No inflammation of any kind	85

TABLE 7.

RELEASE FROM QUARANTINE.

A. Number of specimens sent to obtain release from quarantine:	
1 specimen	891
2 specimens	627
3 "	142
4 "	69
5 "	18
6 "	14
7 "	6
B. Release from quarantine in no. of days after beginning of disease:	
1–14th day	700
15–21st "	654
22–28th "	279
29–35th "	4
36th "	4

Table 1 proves exceedingly interesting and shows that diphtheria bacilli were found in 60 per cent of cases in which the clinical diagnosis of diphtheria was made; in 15 per cent of the cases in which the diagnosis of non-diphtheritic inflammation of the tonsils, pharynx, larynx, or nares was made; in 36 per cent of the cases in which the physician made a diagnosis of "possibly diphtheria;" and in 38 per cent of the cases where the diagnosis was questionable in the mind of the physician. It may seem strange that diphtheria bacilli were found in only 60 per cent of the cases in which a clinical diagnosis of diphtheria had been made. This rather low percentage is probably due, to a great extent, to the fact that many of the pseudo-membranous forms of angina caused by streptococci, staphylococci, influenza bacilli, pneumococci, colon bacilli, and the spirochete of Vincent's angina present a picture clinically often indistinguishable from true diphtheria, and for that reason such cases have in many instances been called diphtheria. In many of these cases repeated subsequent examinations have confirmed the first findings. The figures of the Department of Health of the city of Chicago, as given in its biennial report for the year 1904–5, show that diphtheria bacilli were found in only 37 per cent of cases that were clinically diagnosed as diphtheria, in 16 per cent of cases clinically diagnosed as non-diphtheritic, and in 28 per cent of cases in which the clinical diagnosis was not given. In making these calculations it must be remembered that there is a certain percentage of error—an error of technique. Just how great this is we have never been able thoroughly to satisfy ourselves. Our table further emphasizes the fact that the clinical features of diphtheria are such that the laboratory finding is of inestimable value as

an aid in obtaining a correct diagnosis. The greater discrepancy between the laboratory and clinical findings, as seen in the reports from cities as compared with the figures which we give, may be accounted for by the fact that in cities diphtheria is more or less constantly present. A knowledge of this, together with the constant fear of the possibility of epidemics, causes the city physician to report many cases of simple inflammation of the throat as suspected diphtheria.

Table 2 brings out the fact that 1,203 out of 1,938 cases of diphtheria occurred among school children or among students, and 27 cases among teachers. This may, of course, be explained in large part by the fact that diphtheria occurs more frequently among children. It also, however, suggests the part played by the schoolroom in transmitting disease—a part which is still more emphasized by consideration of Table 4. The rather large number of cases occurring among physicians and nurses, as compared with people in other professions and occupations, is in accordance with what has long been recognized, that diphtheria is one of the most frequent and fatal diseases affecting medical attendants. The fact that in a number of cases physicians and nurses have been found to carry the infection to their patients emphasizes the necessity of the utmost precaution against carrying infection which should be taken by them when visiting and examining diphtheria patients. The use of a cap and gown which may be disinfected after each visit is recommended.

When we consider that in 1,453 out of 2,038 cases under observation as shown in Table 4, the source of infection was not known, we must conclude either that diphtheria bacilli may survive some time on various substances, such as dirt and dried sputum, which experiment has proved is rather improbable, or that infection is due to so-called "bacilli carriers"—people who may or may not have had a previous attack of the disease, but who carry in their throats diphtheria bacilli of sufficient virulence to cause infection in other individuals.[1] This emphasizes the importance of rules prohibiting expectoration in public, which is no doubt a fertile source of such unknown infections; also the importance of repeated bacteriological examinations to determine the absence of virulent diphtheria bacilli before the release of exposed individuals from quarantine.

[1] For references on this subject see footnote: *Jour. Infect. Dis.*, 1907, 4, p. 36.

In 395 out of 2,038 cases infection was traced directly to the schoolroom. Proper and routine medical inspection of school children, especially when diphtheria is known to exist in a community, is thus exceedingly important. When the disease is at all prevalent among school children, the schools should be closed. I also believe it to be advisable that schoolrooms should be occasionally disinfected when diphtheria exists in a community, because of the danger from the so-called "bacilli-carriers."

It should be said that the cases reported as having no inflammation represent, in a number of instances, members of a family who had been exposed to the disease in which the general symptoms led the physician to suspect the possibility of diphtheria, but where no inflammation existed to account for any local infection.

In the majority of instances, 1,505 cases, no antitoxin had been given at the time the specimen was taken for examination, while in 815 cases it was given. This does not, of course, mean that in all of those instances the physician waited for the report from the laboratory before giving antitoxin. We have found many instances in which no antitoxin was given until the laboratory report was received. This may be considered advisable in some cases when symptoms are very mild. We have always urged, however, that in all cases where diphtheria is suspected and the disease is at all marked, physicians should give antitoxin immediately, the earlier the better, and not wait until the laboratory report is received. During that time the patient may be isolated, and if developments, based upon the laboratory and clinical findings, should prove that diphtheria does not exist, such patient need not of course be quarantined. Instances have occurred in which the patients were charity cases, and physicians, knowing that the cost of the antitoxin must be borne by themselves, have not given the serum until the laboratory reported positive findings. This procedure can scarcely be considered as entirely proper in the interest of the patient, nor yet can the physician be blamed so long as the official authorities continue to cut or refuse to pay bills for charity patients. Provision should be made for the proper compensation of physicians doing charity work; or, better yet, antitoxin should be furnished free to charity patients.

As stated, patients may be released from quarantine when two con-

secutive examinations of swabs from both the nose and throat indicate the probable absence of diphtheria bacilli. It will be noticed (Table 7A) that many patients have been released on one examination. Many of these represent cases of members of families who have been exposed to diphtheria and who have been released from quarantine upon a single examination taken after a period of incubation of seven days. The period of incubation should be taken into consideration, inasmuch as we cannot necessarily expect to find diphtheria bacilli during that period, and many patients who might later develop the disease would be released from quarantine through a negative result based upon a bacteriological examination within that time. In many instances also the physician has not followed the rule of two consecutive examinations to the letter and has allowed the release upon a single examination. It will readily be understood that in rural communities a strict observation of the law cannot always be maintained. Our table indicates that, in a number of cases, three, four, five, six, and even seven specimens were necessary to obtain release from quarantine. These numbers may be accounted for by the fact that either physicians begin to take the specimens for release very early, and take them in rapid succession, or that the bacteria remain in the throats of these patients an unusually long time.

The old-time rule enforced in this state was that all cases of diphtheria must remain in quarantine for five weeks. This has now been modified to read as follows:

> In districts where it is not possible or desired to use the laboratory findings as a means of regulating quarantine, those suffering from diphtheria shall be quarantined for a period of not less than four weeks from initial symptoms where antitoxin is used, and five weeks where antitoxin is not used.

Table 7 also shows that 700 out of 1,682, or 41 per cent, were released from quarantine before the end of the second week after the beginning of the disease; 654, or 32 per cent, before three weeks after the beginning of the disease; 279, or 16 per cent, from three to four weeks after the beginning of the disease; 45, or 0.02 per cent, from four to five weeks after the beginning of the disease; and that 4, or 0.002 per cent, were not released from quarantine until after five weeks after the beginning of the disease. The large number of cases of release from quarantine within two weeks after the beginning of

the disease may be accounted for in part by the number of specimens taken from individuals exposed to the disease, but released from quarantine as soon as a negative report was received from the laboratory; in part by the fact that in a number of cases diphtheria bacilli had entirely disappeared from the nose and throat before the end of the second week after the beginning of the disease; in part, however, it must also be explained by the fact that in a number of cases physicians have taken advantage of the laboratory method of releasing patients from quarantine, and have so collected the specimens for examination that a negative report was bound to be received, whether diphtheria bacilli were present or not. The rule enforced in some of our larger cities (Des Moines is the only city in this state following such rule) is that the health officer or medical inspector must take all of the specimens for release from quarantine, or at least the last specimen after the physician has received one negative report from the laboratory. This rule is not easily enforced in many of the smaller towns and rural communities. Because of the fact that certain physicians will take advantage of this privilege, the board has passed the following resolutions:

It is recommended that no culture for release from quarantine be taken prior to fourteen days from the date of the development of the disease.

A second culture for release from quarantine should not be taken until report of first culture is received.

The table is, however, of significance in that it shows that a majority of the cases of diphtheria have been released from quarantine during the second and third week after the beginning of the disease process. When examinations prove the presence of the diphtheria bacilli after five weeks from the date of development of the disease, the virulence test of the microorganism is always made to determine whether the bacteria present are or are not virulent. If found not virulent, the patient may be released from quarantine.

ON THE SO-CALLED PHYSICAL CHEMISTRY OF HEMOLYTIC SERUM.*†

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A FEW years ago, there were assigned to certain serum phenomena a number of physico-chemical laws. It is immaterial for our present purpose what these laws are. Their importance, however, to the future of experimental medicine is evident from the fact that, if true, they not only furnish the first accurately demonstrated facts regarding the molecular composition of certain immunity substances, but they give medical science an instrument by means of which the exact chemical composition of these most important substances might eventually be worked out.

What is apparently the simplest of the phenomena to which these laws were applied, is the phenomenon of the absorption of hemolytic amboceptor by blood corpuscles. It is apparently a simple thing to expose washed corpuscles to accurately measured quantities of heated hemolytic serum, to allow them to stand in contact for definite periods of time, and then to centrifuge the serum free from corpuscles and determine by analysis the amount of unabsorbed amboceptor remaining in it.

In attempting to verify the proposed law,[1] however, numerous experimental difficulties were encountered. Contradictory and even paradoxical results were obtained.[2] These findings were explained by the subsequent discovery that heated hemolytic serum is so altered by contact with corpuscles as to render it unanalyzable by direct quantitative methods.[3]

If one is given an HCl-solution of unknown strength, and is asked to determine its strength in terms of a standard HCl-solution, it is a

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† Presented before the joint meeting of the Society of American Bacteriologists and the section on Physiology and Experimental Medicine of the American Association for the Advancement of Science, at New York City, December 28, 1906. Work aided by the Rockefeller Institute for Medical Research.

[1] For technique, material, etc., see *Jour. Infect. Dis.*, 1905, 2, p. 461.

[2] *Jour. Infect. Dis.*, 1905, 2, p. 485; *Centralbl. f. Bakt.*, 40, p. 383.

[3] *Jour. Infect. Dis.*, 1905, 2, p. 493; *Centralbl. f. Bakt.*, 40, p. 386; *Jour. Biol. Chem.*, 1905, 1, p. 213.

very simple matter to titrate samples of the unknown solution, and calculate its strength as a certain percentage of that of the standard solution.

Suppose, however, that a 4 c.c. sample is taken, and that it is found by titration to contain apparently 20 per cent as much HCl as an equal volume of the standard solution. Suppose, further, that a duplicate titration with a 3 c.c. sample gives apparently 40 per cent; a third titration, with 2 c.c., 60 per cent; a fourth, with 1 c.c., 75 per cent; and a fifth, with $\frac{1}{2}$ c.c., 90 per cent. We would say that the unknown solution was quantitatively unanalyzable, because duplicate titrations do not agree.

This hypothetical result is difficult to imagine, when one is dealing with simple, inorganic substances. It is exactly the kind of result, however, that is obtained when one attempts to determine the residual amboceptor in exposed heated hemolytic serum. Duplicate analyses do not agree, and differ as widely as the results indicated in the hypothetical problem above. The exposed serum is quantitatively unanalyzable.

What is the cause of this phenomenon?

Heated hemolytic serum contains, not only amboceptor, but other substances as well. These are substances that are either originally present in the serum, or that are formed during the heating necessary to destroy the complement. These substances, for convenience, have been spoken of as the "third serum component."[1]

Investigation shows that the third component may at times be antilytic, or hemolysis-inhibiting, in its action, and at other times auxilytic, or hemolysis-increasing. Also, that the third component differs in different animals and under different experimental conditions, and that it apparently consists of a mixture of a number of quite distinct substances, each with its specific effect on hemolysis.

It is found that the third component is altered by exposure to corpuscles. A third component that originally exercises a stimulating action on hemolysis has that action decreased by such exposure, or even replaced by an inhibiting action. And a third component that originally exercises an inhibiting action has its inhibiting action increased.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 647.

Two hypotheses can be put forward to account for this change in the third component; first, that certain substances are absorbed from the third component by the corpuscles during such exposure; second, that the corpuscles give off into the third component substances that influence its action. Both hypotheses were tested.

In order to determine whether or not substances are absorbed from the third component, washed corpuscles were exposed to a third component having the maximum hemolysis-increasing power; they were then washed free from the serum and their susceptibility to hemolysis compared with that of unexposed corpuscles. No change was found in susceptibility after such exposure. The third component, therefore, is either not absorbed by corpuscles, or, if absorbed, is held in such a loose chemical union that it is easily removed by successive washings. Absorption is experimentally undemonstrable.

To determine whether or not substances are given off by corpuscles into the third component, washed corpuscles were exposed to physiological saline, under conditions identical with those of the absorption experiments above, the salt solution simply taking the place of the heated serum. The physiological saline was then freed from corpuscles by centrifugation, and its effect on hemolysis tested.

In all cases, it was found that the exposed salt solution was strongly antilytic. Corpuscles, therefore, give off an hemolysis-inhibiting substance into the third component. Whether or not, this accounts for the total change in exposed third component has not as yet been determined.

Reverting now to the original problem of testing the physico-chemical law proposed for the absorption of hemolytic amboceptor, we see that when heated hemolytic serum is exposed to corpuscles, three phenomena occur: first, an as yet purely hypothetical absorption of amboceptor; second, a demonstrable change in the third component; and, third, a giving-off into the serum of antihemolytic corpuscle products.[1]

No direct measurement, therefore, of the amboceptor-power of exposed serum will give any idea whatever of the amount of amboceptor remaining in it. Experimental proof or disproof of the proposed physico-chemical law is therefore at present impossible.

[1] The second and third changes may, of course, be identical.

Similarly, the presence of an active third component, which differs in different sera prepared under identical conditions, prevents the experimental verification of the physico-chemical law proposed for the interaction of hemolytic complement and amboceptor. *The physical chemistry of hemolytic serum is, therefore, beyond the present reach of experimental science.*

THE TRYPANOSOMES OF MOSQUITOES AND OTHER INSECTS.* †

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ISOLATION OF TRYPANOSOMES FROM MIXED CULTURES.

SUMMARY.

REFERENCES.

EXPLANATION OF PLATES.

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†A preliminary note on this investigation appeared in *Science*, 1906, 23, p. 207, and in the *Jour. of Hyg.*, 1906, 6, pp. 110, 111; also in the *Bull. de l'Inst. Pasteur*, 1906, 4, p. 243, and in the *Centralbl. f. Bakt.*, 1906, 38, p. 326.

‡This study was carried on during the summers of 1905 and 1906, and was rendered possible by the generous aid given by the Board of Directors of the Rockefeller Institute.

INTRODUCTION.

In the paper on "Bird Trypanosomes"[1] we expressed the opinion, in view of the ease with which trypanosomes can be cultivated in the test-tube, that it is reasonable to suppose the same result may take place in the mosquito. That is to say, the few trypanosomes which chance to be present in the blood sucked up by the mosquito may rapidly multiply in the stomach of the insect and give rise to rich cultures similar in many respects to those met with in the test-tube. This view, based entirely upon the cultural characteristics of the bird trypanosomes, has an immediate bearing, not only upon the question of the transmission of trypanosomes, but also upon that of the relation of flagellates to certain intracellular parasites, as claimed by the late Dr. Schaudinn, and for that reason it was desirable to obtain, if possible, an experimental confirmation.

The obvious procedure would be to allow mosquitoes which had been raised from eggs in the laboratory, to feed on birds having a pure trypanosome infection. On subsequent examination such mosquitoes should show cultural forms in the gut, if the above view was correct. Unfortunately, on account of the difficulty of procuring and keeping alive wild birds the choice of birds for these experiments was limited to the sparrow and canary. In our previous work we had found trypanosomes in sparrows, but only in a very small percentage of the birds examined. We hoped, however, by means of cultures of the bird trypanosome to secure pure infections of sparrows and canaries, and with this object in view, a large number of sparrows were examined during the summer of 1905, microscopically and culturally, but with negative results. The failure to obtain infected sparrows and the inability to work with *Tr. paddae* which, as shown by Thiroux, is admirably adapted for laboratory infection, compelled us to abandon this plan and resort to a different procedure.

The rather common occurrence in birds of trypanosomes, and of cytozoa, suggested a study of the flagellates in "wild" mosquitoes. If our view was correct, then it might be possible to find in the gut of such mosquitoes cultural forms of bird trypanosomes. As will be shown, flagellate infection of mosquitoes is quite common, but the forms met with do not correspond to any one of the numerous strains

[1] *Jour. Infect. Dis.*, 1905, 2, p. 303.

which we cultivated from birds. Previous observers had already described such organisms in the gut of insects, but their source or nature was far from being understood. They might be derived (1) from the trypanosomes of the blood of birds or other animals; or (2) they might be stages in the life-cycle of cytozoa, as held by Schaudinn; or (3) lastly they might be harmless, non-pathogenic parasites peculiar to the insect and in nowise related to either of the two types mentioned above.

It is plain that each of these three possibilities must be considered in order to arrive at a definite conclusion regarding the flagellates found in the gut of sanguivora, such as mosquitoes, flies, fleas, lice, leeches, etc. A mere morphological study is incapable of deciding for certainty between the three conditions. Even when supplemented by animal inoculations, effected either by the bite of the mosquito, etc., or by injection of suspensions of the insects, the result is still open to question. For it is conceivable that the mosquito or other host may have a double or even triple infection, and hence the result obtained (i. e., appearance of cytozoa) may be due to an unrecognized stage and not to the flagellates actually seen. The recent work of Edm. and Et. Sergent on the transmission of the halteridian infection of pigeons affords a demonstration of the existence of such a stage. It is true that in proteosoma infection, and in human malaria this method of investigation has given most satisfactory results, but when applied to flagellate studies it may prove, and undoubtedly has proven, misleading. The relation of cause and effect in such problems can be brought out with the least uncertainty by the aid of pure cultures of the trypanosomes in question, and this method of study, so well established in bacteriology, should be applied as far as possible to the solution of questions in protozoology. By this means we have already shown, contrary to Schaudinn, that the trypanosomes of birds are in nowise related to the hemocytozoa. The applicability of this method of research to insect trypanosomes will be seen from the results of this study.

Our investigations upon the mosquito trypanosomes were made during the summers of 1905 and 1906. They show that the flagellate infection of mosquitoes is of common occurrence, and that several distinct species are to be found. It has been possible to grow these

organisms in mixed and even in pure culture, and to show that they are closely related to, and indeed actually are, true trypanosomes. The forms which have been obtained in the culture-tube are similar to, and even identical with, those found in the digestive tube, thus showing that the forms met with *in vivo* are in reality cultural forms, as we have contended. This fact goes to show the absurdity of the contention that cultural forms represent degeneration or involution types. The cultivation method has enabled the differentiation into two species of forms heretofore regarded as belonging to one organism. Furthermore, the inoculation of various birds and mammals with such cultures was not followed by the development of intracellular parasites, or by the appearance of trypanosomes in the blood, which would seem to indicate that the mosquito flagellates are parasites peculiar to this insect.

PREVIOUS OBSERVATIONS.

Before proceeding to a detailed account of our work, it is desirable to present, as briefly as possible, a résumé of the observations which have been made up to the present time regarding the presence of flagellates in the guts of various insects and also of leeches. It will be seen that flagellate infection of the digestive tube is not limited to the sanguivora, but that it may occur in insects that do not feed or have not fed on animals.

The earliest observations upon the presence of flagellates in mosquitoes were made by Ross, in 1898, in India. Recently (1906) he has called attention to these early studies, and there can be no doubt but that the forms seen by him were the same as those which Léger subsequently described as *Crithidia fasciculata*. In various species of *Culex*, principally *C. fatigans*, he found "sporangia" or radiating clusters (rosettes) of small active bodies, which under water separated into thousands of flagellulae. These were about 8 μ by 2 μ in size, with numerous minute black points in their substance, and had a single long flagellum. Another form, which he designated as amoebulae, he now considers as transitional, dividing forms corresponding to the truncated *Crithidia*. Apparently, the long *Herpetomonas* which will be described later was not encountered. The forms mentioned were also observed by him in *Anopheles*, but not in *Stegomyia*. They occurred in the larva, pupa, and imago; at times, in a considerable proportion of the insects examined. It is interesting to note that Ross during his work on Proteosoma found the flagellates but once in mosquitoes, although these were fed on birds many of which contained Halteridium as well as Proteosoma. This fact, as well as their presence in all three stages of the insect, goes to show, as Ross points out, that they had been already present in the insects before these were fed on the blood. He very correctly concludes that there is little reason

for supposing that the *Crithidia* are developed from cytozoa, and he expresses the belief that they are derived from organisms contained in the intestines of the larva and probably swallowed by it. He further points out the probability that Schaudinn's mosquito flagellates were merely *Crithidia* which were already present in the insects with which he worked.

The next observation is that of Durham, who, in 1900, examined a specimen of *Stegomyia fasciata* which had fed the night before on a small bat. The blood in the mosquito contained abundant trypanosomes whose shape was quite different from the usual ones in rats, nagana, etc. The presumption was that these trypanosomes came from the bat, but this, owing to its death and rapid decomposition, could not be verified with certainty. Although flagellates, coccidia-like bodies, etc., were found from time to time in the eighty mosquitoes which were dissected, this was the only instance in which trypanosomes were found. Nothing further is stated regarding the flagellates mentioned.

Chatterjee, in 1901, while dissecting different *Anopheles*, found in the abdominal cavity of one of them an organism which appeared to him to be closely related to the trypanosome of surra. It was actively motile and possessed a fine flagellum, longer than the body, and an oval nucleus. Not having access to the original paper, it is impossible to say whether this form belonged to *Crithidia* or *Herpetomonas*.

In the same year Christophers noted the presence of swarms of a flagellate organism in the rectum and throughout the hind- and mid-gut, in a large proportion of *Anopheles* and *Culex*. The illustration given by him (Ref. 13, Fig. 3, Plate V) is suggestive of *Crithidia*. The presence of these parasites is also mentioned by Stephens and Christophers.[62] In this connection it may be stated that from recent private communications of Captain Patton and also of Captain Christophers we learn that a large percentage of mosquitoes at Madras have *Herpetomonas* and *Crithidia*. Christophers writes that he has found developmental or immature forms of *Herpetomonas*, clustered about the pylorus, in the larvae of *C. fatigans*.

An important study on the flagellates of mosquitoes was published by Léger in 1902. In the gut of *Anopheles maculipennis* he found a peculiar flagellate, presumably the same as that observed previously by Ross and Christophers, which on account of certain characteristics he placed in a new genus, to which he gave the name *Crithidia*. He classed it among the Cercomonadina, intermediate between *Herpetomonas* and *Trypanosoma*. The distinction between the two genera, *Herpetomonas* and *Crithidia*, was based largely upon the difference in size of the adult, free, or monadian forms. It will be shown presently that Léger failed to recognize the possibility of double infection in insects, and that he has at times included in one description two distinct species.

In his critical study of the blood flagellates, Lühe points out that the difference in size of the adult, free, or monadian forms is hardly sufficient to justify the creation of a new genus. Lühe, however, would characterize the genus *Crithidia* as possessing a single flagellum with the blepharoplast situated near the nucleus, whereas the true *Herpetomonas* has a double flagellum (as described by Prowazek for *H. muscae-domesticae* and *H. sarcophagae*) and a terminal blepharoplast. This characterization of the two genera is open to question, as will be shown later on. It should be borne in mind, however, that the distinctions between *Trypanosoma*, *Herpetomonas*, and *Crithidia* are at the best highly fragile.

It is interesting to note that Léger raised the question, in view of the way in which *Anopheles* fed, and the analogy of the elongated forms to trypanosomes, whether the

Crithidia did not represent a developing stage of some flagellate hematozoon, more especially the trypanosomata of vertebrates. This question is as yet unanswered, although it is probable that the *Crithidia* are parasites peculiar to the mosquito and are not immediately derived from the trypanosomes in the blood of other animals. As will be seen, Schaudinn has endeavored to show that these, or very similar forms, represented stages in the development of the *Halteridium*.

Léger designated the flagellate found by him in Anopheles as *Crithidia fasciculata*. He described two types with transitional forms one to the other. The first, or gregarine-like, or resting form, is more frequent. It appears as a short, thick oval, resembling a grain of barley. It is slightly flattened, and the anterior end, which carries a whip as long as the body, is truncated. These smaller forms measure 3–4 μ in length, but may attain 6–8 μ. The anterior end is slightly curved or may be indented. The body is hyaline or faintly granular, and at times shows one or more clear spots resembling vacuoles. The whip starts at the centrosome or blepharoplast which lies in front or to the side of the nucleus. The division is longitudinal and corresponds to that of trypanosomes. Rosette formation is common, and the small cells may be united in masses or in radiating bundles which are attached to the intestinal wall.

The second, termed the monadian or free form, is an elongated cell, 8–14 μ in length, and resembles exactly small trypanosomes. The anterior pointed end is drawn out along the whip, and the cell shows a rudimentary undulating membrane. As mentioned above, Léger held that the length of the free form afforded the chief distinction between this genus and that of *Herpetomonas*.

Léger and Duboscq in the same year found this organism, either as the free cells or in massive rosettes, in the intestines of *Anopheles* larvae, as well as in hibernating adults.

The work of Schaudinn, in 1904, is of especial importance, for, no matter what may be the final interpretation of his observations, it has served to call attention to the flagellates present in the digestive tract of insects and other sanguivora, and in so doing has stimulated inquiry, not only as to the relation of these forms to the blood protozoa, but also as to the mode of transmission of the pathogenic protozoa in general. As is well-known, he described the formation of trypanosomes and so-called spirochetes in the common mosquito, *Culex pipiens*, which had previously fed on owls infected with intracellular organisms (Halteridium and Leucocytozoon) and trypanosomes. In view of the the fact that a summary of this extensive paper was given in our paper on "Bird Trypanosomes," it is hardly necessary to repeat it here. It was pointed out in that paper that the mosquito flagellates of Schaudinn were probably derived from the bird trypanosomes which had multiplied in the digestive tube of the insect. As will be seen, the present study offers another and more plausible explanation of the origin of Schaudinn's flagellates.

It is important to note, as Schaudinn himself has pointed out, that the forms of *Trypanosoma noctuae*, as found and described in the mosquito, show the greatest resemblance to the *Crithidia* of Léger. Indeed, because of the gregarine-like, resting condition and the mode of multiplication, he inclined to the belief that the *Crithidia* represented a developmental stage of a closely related parasite. Notwithstanding the fact that Schaudinn recognized this similarity, there is no evidence, as Ross rightly asserts, that he excluded the presence of *Crithidia* in the mosquitoes used in his experiments. It is a striking fact that, while he refers to Léger's observation on *Crithidia*, he makes no mention of the presence of the same or similar forms in the *Culex pipiens* with which

he experimented, and which were presumably captured at large. From what is now known regarding the distribution of *Crithidia* and *Herpetomonas* in mosquitoes, we must recognize a grave source of error which Schaudinn apparently failed to take into consideration. It will be remembered that he was able to find trypanosomes in only about 10 per cent of the mosquitoes used in his experiments. As will be seen, we have found flagellates in as many as 15 per cent of the mosquitoes captured during one season. This fact itself is very suggestive of the nature of the organisms observed by him. It is our belief that Schaudinn's *Tr. noctuae* is in nowise related to the owl halteridium, and that it either corresponds to *Crithidia fasciculata* or to a mixed *Crithidia* and *Herpetomonas*. Furthermore, the *Spirochaeta Ziemanni* which he later recognized as a trypanosome and not a true spirochete, would seem to correspond to a *Herpetomonas* like our *Tr. culicis* or to *Tr. avium* were it not for the recent observation of Töpfer regarding the presence of spirochetes in owls. This work, as will be shown later, throws new light upon the nature of *Sp. Ziemanni*.

In the same year (1904) Edm. and Et. Sergent, working in Algeria, repeated the work of Schaudinn and obtained essentially the same results.[1] That is to say, flagellates were found in 28 per cent (14 out of 50) of *Culex pipiens* which were examined 36–48 hours after having been fed on an owl (*Athene noctua*), infected with halteridia. This large percentage of infected mosquitoes, as compared with Schaudinn's 10 per cent, is of no special significance, since we have repeatedly met with small batches of "wild" mosquitoes in which one third or more showed flagellates. The Sergents state explicitly that they had never met with similar forms in the stomachs of mosquitoes which had not fed on infected blood.

This general statement, in view of the widespread natural infection of mosquitoes, is difficult to understand unless it be assumed that these control insects were examined directly—that is, without having been fed upon a clean animal. It is perfectly obvious that a rigid control test demands that the mosquitoes be fed upon the blood of a clean, or non-infected bird, or even mammal, in order to allow the flagellates, if any are present, to multiply. The fact that the mosquitoes used in these experiments were raised from larvae in the laboratory does not, as is now known, exclude a natural flagellate infection of the adult insect. This fact they themselves recognized in the following year, as will be shown presently.

With the mosquitoes which had fed upon the infected owls the Sergents were able to produce a halteridium infection in young owls which had been raised in the laboratory and were presumably free from parasites. Thus, an owl injected with the stomach contents (containing trypanosomes) of a mosquito that fed on an infected bird two days before, developed halteridia 11 days later. Similarly, a second owl which received the stomach contents of two mosquitoes showed halteridia three days

[1] The forms which the Sergents identified as "indifferent" trypanosomes measured 25 μ in length by 3 μ in width. The larger ones were 36 μ long. This is given as the total length including the flagellum, the latter being as long as the body. The "male" trypanosomes were small, on an average 14–15 μ in length, and this presumably includes the whip. The deeply staining, granular "female" forms were rare. They note a slight difference between their trypanosomes and those of Schaudinn in that their forms were "plus effilés, moins renflés" than those figured by the latter. This fact, taken into consideration with their description of the indifferent, male and female, forms, makes it probable that they were dealing with *Herpetomonas*, probably the same as our *Tr. culicis*. On the other hand, Schaudinn's figures (Figs. 1, 2, and 4) show a rounded posterior end, and in this respect resemble *Crithidia;* in other regards, like the presence of an undulating membrance and posterior bodies, they suggest a *Herpetomonas*. The *Herpetomonas* only rarely shows an enlarged, rounded posterior end, as will be shown later in connection with the involution forms of *Tr. culicis*.

later. On the other hand, a third owl which received an injection of the stomach contents of three mosquitoes failed to become infected (!). In another experiment six mosquitoes, fed a month before on an infected bird, were allowed to bite a young owl, which soon developed an infection. Two other owls bitten by these same mosquitoes or by others, failed to show halteridia.

The Sergents looked upon their work as a crucial experiment supporting Schaudinn's view as to the relation of *Tr. noctuae* to the halteridium. It must be confessed, however, that these tests were by no means as rigid as they supposed; for, although they employed mosquitoes raised from the larval stage and made use of young birds taken from the nest, and found presumably to be free from infection, they clearly overlooked several possible sources of error. In the first place, the owls upon which the mosquitoes fed may have been infected with trypanosomes as well as halteridia. They themselves recognized the presence of a trypanosome in 1 out of the 3 or 4 infected owls with which they worked, and it is not unlikely that this flagellate was present in the others, though in very small numbers. It is therefore possible that the mosquitoes which fed upon these owls developed a double infection, trypanosomal and halteridian, the latter being readily transmitted by an unrecognized stage, whereas the former gave rise to a "culture" of the flagellates in the digestive tube. An error of this kind can be obviated by demonstrating that the birds are *culturally as well as microscopically* free from trypanosomes.

A second and perhaps even more important factor which must be taken into consideration is the fact of the not infrequent presence of trypanosomes in the larval and pupal stages, and hence in adult mosquitoes before these have fed upon the infected bird. It is evident that the use of mosquitoes which have been raised in the laboratory from the eggs does not insure the exclusion of flagellate infection of the intestinal tract of these insects.[1] This fallacy can be guarded against by means of a control experiment in which a large enough number of "raised" mosquitoes are fed upon non-infected animals. In this way it should be possible to show whether or not the percentage of flagellate infection is greater in the mosquitoes which fed upon infected birds, and whether such flagellates are identical with or different from those naturally present in the stomach.

A third and equally important objection is based upon the apparent absence of control inoculations with the stomach contents, free from trypanosomes, of mosquitoes fed on infected birds. In Schaudinn's work 90 per cent of the mosquitoes thus fed showed no flagellates, while with the Sergents the percentage was not as high, only 72 per cent. What would be the result if the stomach contents of these mosquitoes was injected into clean owls? There is no record that such experiments were made; and yet the need of a control test of this kind is perfectly obvious; for, if the halteridian infection is due to something other than flagellates, it should be possible to secure positive results with such trypanosome-free mosquitoes.[2] On the other hand, the failure

[1] As will be seen from their work of the following year, reference to which is given below, they succeeded in finding a *Herpetomonas* in 11 per cent of mosquitoes raised from the larval stage in the laboratory.

[2] The possibility of the presence of an unrecognized stage of halteridium in the mosquitoes is seen from the recent work of the Sergents on the transmission of this parasite to pigeons. After feeding some Hippoboscides (*Lynchia maura*) on infected pigeons in Algeria, the insects were taken to Paris and placed on clean pigeons. The latter became infected after a period of incubation of 34–38 days. Intravenous injections of suspensions of *Lynchia* caused infection after 28–29 days; and a Berkefeld filtrate infected after 36 days. This long period of incubation, when compared with that noted in owls after injection of mosquito trypanosomes, lends color to the belief that these birds had an unrecognized latent infection. Trypanosomes were apparently not present in the infected *Lynchia*.

to obtain infection in such an experiment would be an argument in favor of Schaudinn's views.

Another source of error which must be rigorously guarded against is the possibility of the existence of a latent halteridian infection in the owls seemingly free from parasites. The infection of owls and other birds very commonly takes place before they leave the nest. This fact was first pointed out by Danilewsky, and we have repeatedly made similar observations. Hence, nestlings cannot be said to be free from infection unless repeated examinations are made *extending over a period of several weeks* to allow for the maximum period of incubation. A very good illustration of latent infection is sometimes observed in sparrows which receive an injection of proteosoma. Although at first apparently free from parasites, they may develop halteridia in the course of a week, along with the proteosoma. The halteridia were not introduced by the injection, for it is not possible to infect birds in that way. The statement of Schaudinn that the halteridian infection of owls can be transmitted from owl to owl by injections of the infected blood, "was ja schon lange bekannt ist," is open to question, as previous workers, employing, it is true, other kinds of halteridia, have not been able to induce an infection by means of blood injections. In view of this fact, it is probable that the apparently successful inoculations with infected blood (and with mosquitoes) may be due at times to the lighting-up of a latent infection. (See also p. 240 and footnote p. 259.)

Although unconfirmed, except as mentioned above, still Schaudinn's views regarding the nature of the mosquito flagellates have been accepted by most zoologists. Some, indeed, have gone as far as to interpret the work done on the owl as applying to all trypanosomes, and in this they have gone beyond the position taken by Schaudinn himself; for he realized, as we know from his letters, that there were trypanosomes without an intracellular stage, and that there were halteridia which had no trypanosome stage. As a matter of fact, the whole question has narrowed itself down to the parasites of *Athene noctua* and their relation to the mosquito flagellates. It is not a question of a broad, general biological principle.

The work of the Sergents is of further interest, inasmuch as they found in the Malpighian tubes of mosquitoes (three out of 17) which fed on owls infected with *H. Ziemanni* the spirochetal forms described by Schaudinn. These flagellates, which they describe as being 25–30 μ long and 1 μ wide, are clearly different from the true spirochetes, which scarcely exceed 0.2 μ in width. Similar forms were not found in 52 Culices from the same brood as those used in the experiment; but whether these control mosquitoes were fed on non-infected birds is not stated. Repeated attempts to inoculate a barn owl, *Strix flammea*, either by injection of these flagellates or by the bites of mosquitoes, failed.

The measurements as given by the Sergents for *Sp. Ziemanni* agree perfectly with those of the long Herpetomonad form (*Tr. culicis*) which will be described later. This statement does not necessarily imply that the forms described by Schaudinn correspond to this trypanosome, for, as a matter of fact, his illustrations of *Sp. Ziemanni* (see his Fig. 17) show forms which have the blepharoplast posterior to the nucleus, and hence have a very long undulating membrance. Such is not the case with *Tr. culicis*. The forms mentioned, however, do resemble, remarkably, the free-swimming, long type of *Tr. avium*, as we have shown in the paper on "Bird Trypanosomes" (Plate 10). As to the possibility of the presence of true spiochetes see footnote p. 259.

Still more recently (1906) the Sergents have reported upon the presence of a *Her-*

petomonas in 13 out of 119 (11 per cent) *Culex pipiens* and in one *Stegomyia fasciata* which were raised from larvae in the laboratory. These mosquitoes, it should be noted, though fed either on infected owls or on clean canaries, showed as large a percentage of infections as was obtained by Schaudinn. They designate this organism as *Herpetomonas algeriense*, and describe two forms, motile and non-motile. The motile form was 12 μ long and 2.5 μ wide, and was provided with a whip which was 3.5–6 μ long. The large transverse centrosome was always posterior to the nucleus and about 0.56 μ from the broad rounded end. The non-motile forms were round, about 5.5 μ in diameter, and had a long, free flagellum measuring even 17 μ in length. Although the mosquitoes fed in part on owls infected with halteridia and *H. Ziemanni*, they do not mention finding the same trypanosomes as in the previous year. (See footnote p. 229.)

They also report on the presence of a very narrow flagellate in the digestive tube of the larvae of several *Anopheles* and of one *Culex;* and also of one nymph of *A. maculipennis*. The body averaged 16 μ in length and 3.5 μ in width, and was provided with a long free flagellum which measured 24 μ. They regard this organism as a *Herpetomonas* resembling the *H. jaculum* which Léger found in *Nepa cinerea*, and they suggest that possibly the latter obtains its infection by feeding upon the mosquito larvae. It will be seen from these observations of the Sergents that they have described four kinds of flagellates in mosquitoes, two of which they identify with the forms of Schaudinn.

Summing up the investigations heretofore made regarding the flagellates of mosquitoes, it will be seen that two distinct types have been recognized, *Crithidia* and *Herpetomonas*, and that each of these is represented by one or more species. Exactly similiar forms have been found in the digestive tract of other insects, some of which, it is worth noting, are not sanguivora.

The following list of the species described up to the present time may not be without interest:

Crithidia fasciculata, Léger, 1902	In the gut of adult *Anopheles* and *Culex;* also in larvae and pupae.
" *campanulata*, Léger, 1903	In intestine near Malpighian tubes in larva of *Chironomus plumosus.*
" *minuta*, Léger, 1903	In mid- and hind-gut of *Tabanus tergestinus.*
Herpetomonas muscae-domesticae, Burnett, 1851.	In the common house-fly; also in *Homalomyia scalaris*, *Pollenia rudis*, *Theicomyza fusca* (Léger).
" *Bütschlii*, Kent, 1881	In intestine of *Trilobus gracilis.*
" *jaculum*. Léger, 1902	In mid-gut of *Nepa cinerea*, adult and larvae.
" *gracilis*, Léger, 1903	In Malpighian tubes of larvae of *Tanypus.*
" *Lesnei*, Léger, 1903	In mid-gut near Malpighian tubes of *Dasyphora pratorum.*
" *subulata*, Léger, 1904	In gut of *Tabanus glaucopis* and *Hematopota italica.*
" *sarcophagae*, Prowazek, 1904	In gut of meat-fly, *Sarcophaga hemorrhoidalis.*
" *bombycis*, Levaditi, 1905	In butterfly of silkworm, *Bombyx mori.*
" *algeriense*, Edm. & Et. Sergent, 1906	In gut of *Culex pipiens* and *Stegomyia fasciata.*
Trypanosoma noctuae, Schaudinn, 1904	In gut of *Culex pipiens* as supposed stage of *Halteridium noctuae.*
" (*Spirochaeta*) *Ziemanni*, Schaudinn, 1904	In gut of *Culex pipiens* as supposed stage of *H. Ziemanni.*
" *Grayi*, Novy, 1906	In gut of *Glossina palpalis.*
" *Tullochii*, Minchin, 1906	" " " " "
" *culicis*, N. Sp.	In gut of various culices.
" *Christophersi*, N. Sp.	In gut of dog-tick, *Rhipicephalus sanguineus.*

The unnamed herpetomonas forms observed in *Culex*, *Anopheles*, and *Stegomyia*, and also that met

with in *Stomoxys* by Gray, obviously cannot be included in the above list. This is also the case with a very small flagellate, one-fourth to one-fifth the diameter of a red blood cell, which Ziemann found in the ovaries of *Chrysops dimidiatus*, a biting fly in Camerun. It may be stated further that Rogers has recently designated the human parasite, *Piroplasma donovani*, as Herpetomonas of Kala-azar. The flagellate which Dutton and Todd found in two out of fourteen house mice in Senegambia, and which they compared with *H. Bütschlii*, is probably identical with *Tr. duttoni* which Thiroux later described in the mice of Senegal. It may further be stated that in *Nepa* Léger found a short oval flagellate about 6 μ long, which he designated as *Otomonas tremula*.

The flagellates recently (1905) described in the gut of the sheep louse (*Melaphagus ovinus*) by E. Pfeiffer have been compared with *Crithidia* and *Herpetomonas*. They may be looked upon as "cultural" trypanosomes, as they show the blepharoplast anterior and close to the nucleus, while the undulating membrane is indicated with reasonable certainty in some of the illustrations given, especially in his Fig. 7. The intestinal epithelial cells were covered with rows of the resting forms, and, in addition, typical rosettes were observed. Unfortunately, no examination of the blood of the sheep from which the lice were taken is mentioned, and presumably was not made.

It is a well-recognized fact that *Tr. Lewisi* is carried from rat to rat by fleas and lice. The transmission by fleas was first established by Rabinowitsch and Kempner, although they were unable to detect the flagellates in the fleas by direct examination. In lice which have recently fed on rats infected with *Tr. Lewisi* this organism can be readily seen, and by placing freshly infected lice on a rat MacNeal was able to secure an infection. The fact that similar experiments failed, with lice containing more or less completely digested blood, goes to show that these insects do not play an active part as hosts, but are mere vectors or passive carriers of the parasites in much the same way as in the case of the tsetse fly.

According to the recent work of Prowazek (1905), the rat louse (*Haematopinus spinulosus*) is an intermediary host, inasmuch as he found trypanosomes, though in variable numbers, in almost all of the lice which fed on infected rats. The parasites were usually present in the stomach; twice they were seen in the Malpighian tubules, and even in the circulating blood, and once in an egg. That the flagellates were actively multiplying in the mid-gut was evident by the presence of divisional forms and of large rosettes or groups which covered the lower end of the stomach wall, especially in the region of the Malpighian tubules. The illustrations which he gives (such as Fig. 55) show undoubted "cultural" forms, since the blepharoplast is anterior to the nucleus and the flagella in the rosettes are directed centrally. Crithidia-like bodies with short flagella, as well as smaller involution forms, without whips, were found. The latter forms were either free in the gut or wedged deep between the cells. He described male and female forms, although, as in the case of *Herpetomonas muscae*, the difference between the two was not very marked.

Prowazek identifies these flagellates of the louse with *Tr. Lewisi*, but this is by no means proven, since he was unable to infect rats by placing upon them presumably infected lice. If the multiplying flagellates of the louse actually represent stages of the cultural form of *Tr. Lewisi*, it should manifestly be possible to produce an infection either by injecting the stomach contents of such lice after they had fully digested the blood which they had taken up, or by allowing them to feed upon clean normal rats. Our own experiments, as mentioned above, indicate that the transmission is effected by *lice which have just fed on an infected rat* and not by those which are void of fresh blood. While, therefore, it is quite reasonable to believe that the ingested trypanosomes multiply in the gut, it is clearly not proven. Although we have carefully

examined Prowazek's memoir, we have not been able to find any statement to show that lice taken from non-infected rats were free from these flagellates. Control examinations of this kind are as essential here as in the work upon mosquitoes. In the absence of such controls and of positive infection experiments, the possibility remains open that these organisms are mere intestinal flagellates of the louse in nowise derived from *Tr. Lewisi.*

In support of this view, and to illustrate the need of controls, we may cite some experiments in which mosquitoes were allowed to feed on rats infected with *Tr. Lewisi* and *Tr. Brucei.* In the stomach contents of these insects the original blood trypanosomes could be detected for 24 to 36 hours after the infective feed, as will be shown ater. At times, *Crithidia* and *Herpetomonas* were present in these insects, and, were it not known that these belonged to the mosquito, they might well have been taken to represent multiplication forms of the blood trypanosomes.

As bearing upon the work of Prowazek should also be mentioned the observations of Nabarro and Greig, Gray and Tulloch, and of Koch regarding the presence of flagellates in the stomach of different species of tsetse flies. These organisms were regarded as multiplication forms of *Tr. gambiense* and *Tr. Brucei,* but the later studies of Minchin, Gray and Tulloch, and of one of us (Novy) show quite conclusively that such is not the case, and that these flagellates (*Tr. Grayi* and *Tr. Tullochii*) are mere harmless parasites of the intestinal tract analogous to those met with in mosquitoes. During the past year Koch himself has recognized the absence of any relation between these flagellates and *Tr. gambiense.* He now inclines to the belief that the tsetse flagellates are derived from the trypanosomes in crocodiles, on account of the similarity which they bear to the cultural forms of the latter.

Another instance of the presence of flagellates in insects is a recent observation made by Balfour (1906). While searching for a developmental cycle of *Hemogregarina Balfouri* in the flea (*Pulex cleopatrae*) which infects the Sudan jerboa, he found a flagellate which occurred in rosettes, or in slightly active forms, and as non-motile gregarines or amoebulae. He regards these organisms as being probably closely related to Léger's *Crithidia fasciculata,* and he further considers it likely that they are "merely parasites of the flea itself" and are unrelated to the hemogregarines of the jerboa. The fact of their presence in fleas fed on non-infected gerbils is important evidence on this point, and is in accord with the observations already noted.

It may further be said that Pricolo has found trypanosomes in fleas taken from mice (*Mus musculus*) which were infected presumably with *Tr. Duttoni.* The significance of the trypanosomes found in cattle and dog ticks will be discussed later.

While on a priori grounds one might be led to believe that the trypanosomes of vertebrates multiply in the digestive tract of insects, the fact remains that up to the present time no positive evidence has been brought forward in support of that view. There is, of course, no doubt as to the fact that trypanosomal infections are spread through the agency of insects, but these seemingly act the part of mere vectors and not of active hosts. It is probable that an exception to this statement is found in the case of kala-azar of India. The parasite of this disease, known as *Piroplasma donovani,* according to private communication from Captain Patton, develops in the gut of the common bedbug into flagellate forms, which then multiply and resemble the *Crithidia* of mosquitoes. This, it may be added, is apparently the only instance apart from the work of Schaudinn on the parasites of the owl, where an intracellular (leucocytic) parasite actually develops into a flagellate stage.

On the other hand, there is one group of organisms, the leeches, where the evidence as to the multiplication of trypanosomes in the gut is quite incontestable. The presence of flagellates in leeches (*Piscicola* and *Pontobdella*) was first noted by Leydig (1849) and later by Labbé in *Haemopis sanguisuga* (15 days after removal from frogs), and it was on account of these observations that Doflein (1901) concluded that the fish trypanosomes were probably transmitted by means of these sanguivora.

Keysselitz was the first to approach the question methodically. Early in 1904 he showed that the leeches were to be considered as intermediate hosts, but the full results of his investigations were not published until 1906. He found a large percentage of leeches (*Piscicola geometra*), captured at large, to harbor in their stomachs variable quantities of flagellates, which because of their morphological characteristics he identified with trypanosomes and trypanoplasmes. Owing to the difficulty of obtaining leeches free from flagellates, he recognized the possible error in employing these in feeding experiments upon fish. He correctly reasoned that, though the fish flagellates would be thus introduced into the leech, it would be impossible to determine whether the multiplication forms observed belonged to them or were derived from the organisms already present in the leech. Hence, in order to obtain unquestionable results, it would be necessary to use leeches that had been raised from the egg and found, by control feeding experiments, to be actually free from flagellates. In his paper he describes, at length, the multiplication forms of trypanoplasmes, but the origin of these is not quite clear. He failed to produce an unquestionable infection by other injections of the stomach contents of the leech or by allowing the latter to feed upon the fish. It is probable that this failure was due to the use of non-susceptible species or such as had an acquired immunity. As will be shown below, Brumpt has succeeded in infecting fish, frogs, and eels by means of leeches.

Billet in 1904 also presented observations going to show that the *Tr. inopinatum* of the Algerian green frog multiplied very easily in the digestive tube of a leech (*Helobdella algira*). Like Keysselitz, however, he was unable to infect frogs either by injection of blood or by the use of infected leeches. Both results, it may be added, have been accomplished since then by Brumpt.

About the same time Léger experimented with loaches which harbor two kinds of flagellates, trypanosomes and trypanoplasmes. The intestinal contents of leeches (*Piscicola*), about four days after these had fed on loaches infected with *Tr. barbatulae*, showed numerous flagellates presenting the three types of Schaudinn. Similarly, leeches (*Hemiclepsis marginata*) placed on fish infected with *Trypanoplasma varium* showed in a few days numerous, small, almost filiform trypanoplasmes in addition to trypanosome-like forms. These observations confirmed the previous results of Brumpt, who noted the presence of prodigious quantities of trypanosomes, showing gregarine and herpetomonad forms, in *Hemiclepsis* taken from fish.

The several studies of Brumpt are of especial importance, as they clearly point to the rôle of leeches in the transmission of flagellates. He brought out the interesting fact that certain species of trypanosomes and trypanoplasmes develop in a given species of leech (*Hemiclepsis marginata*), but not in another (*Piscicola*), while others develop in the latter and fail to do so in the former. He made use of this fact in differentiating a large number of species of trypanosomes and trypanoplasmes. The flagellates found in the stomach and intestines present elongated, herpetomonad, or "cultural" forms, which are sufficiently characteristic to permit the recognition of

species. In these forms, as with most of our cultural forms, the blepharoplast is anterior to the nucleus.

According to Brumpt, the *Tr. granulosum* of the eel when sucked up by the *Hemoclepsis* gives rise in a few hours, in the stomach, to pyriform or crithidia-like bodies, which then pass into the intestine, where they persist in the herpetomonad form for months. Hence he regards the latter as the ancestral form preceding the sanguicolous stage. Such leeches when placed on "clean" eels gave a positive infection in from four to six days. Contrary to Keysselitz, he was able to infect two carps, two chubs (chabots), and one cottus by placing upon these leeches taken from the same species of infected fish.

Similarly, Brumpt has shown that the *Tr. inopinatum* of the Algerian green frog when taken up by a small leech (*Helobdella algira*) rapidly assumes the herpetomonad form, and that such leeches when placed on a frog, even as late as a month after the first feeding, produce a severe and even fatal trypanosomal infection after a period of incubation of from eight to 10 days. Unlike Billet, who, influenced by Schaudinn's work, was inclined to consider the leech trypanosome as a stage of the intracellular *Drepanidium*, he points out that there is no relation between these flagellates and the hemogregarines of frogs.

Lastly, it is in order to emphasize, as Léger has done, the fragile distinctions which exist between *Crithidia*, *Herpetomonas*, and *Trypanosoma*. The first two genera are supposed to have no undulating membrane, and yet a rudimentary one is ascribed by Léger to *H. subulata* and to *C. fasciculata*. On account of the proximity of the centrosome to the anterior end, it is evident that the undulating membrane must be but feebly developed, and hence cannot be readily seen. This is equally true of many of the cultural trypanosomes, and for that reason the failure to recognize the presence of an undulating membrane does not, or should not, exclude an organism from the trypanosome group.

Another difference which has been insisted on is the position of the centrosome or blepharoplast which, in *Crithidia* and *Herpetomonas*, usually lies by the side of, or anterior to, the nucleus, while in the blood trypanosomes it occupies a posterior position. This distinction, however, disappears when the cultural forms of trypanosomes are compared, as they properly should be, with these insect flagellates. Such comparisons are in order, for, as will be shown, the *Crithidia* and *Herpetomonas* present identically the same form in the test-tube as in the digestive canal of the insect. The native flagellates found in the gut of insects, therefore, are cultural forms, and as such are directly comparable with those of the blood trypanosomes. Now, in cultures of *Tr. Lewisi*, *Tr. Duttoni*, *Tr. rotatorium*, *Tr. Mesnili*, *Tr. Laverani*, etc., the blepharoplast occupies a position by the side of, or anterior to, the nucleus, and such forms might very properly be included under *Herpetomonas*. While this position of the blepharoplast is the usual one in cultural forms of trypanosomes, it is not always so. Thus, in some species, notably in the spirochete-like form of *Tr. avium*, it may lie posterior to the nucleus. This position, though rare, has also been observed in insect flagellates, namely *Tr. Tullochii* and *Herpetomonas algeriense*.

It is furthermore worthy of note, showing as it does the close relationship which exists between the cultural trypanosomes and the *Crithidia* and *Herpetomonas*, that all three types give rise to essentially the same gregarine forms. These are attached, whip foremost, to the epithelial cells of the intestinal wall, and are crowded together

in several layers; or they form large rosettes with central flagella or agglomerations filling the lumen of the tube. The polymorphism noted by Léger in the case of *Crithidia* and *Herpetomonas*, and by Schaudinn for *Tr. noctuae*, is equally marked in cultures of blood trypanosomes.

According to Léger, the main difference between *Crithidia* and *Herpetomonas* is in the large size of the adult monadian form of the latter as compared with that of the former. The existence of a double flagellum, as claimed by Prowazek for the *Herpetomonas* of the house-fly, if confirmed, would afford a better generic distinction. Gray, it is true, has found a double flagellum on the *Herpetomonas* of the *Stomoxys*, but it is by no means certain that this condition does not represent an early stage of division.

In view of the cultural characteristics of these three types, we believe it justifiable to consider them as belonging to the one genus *Trypanosoma*. The terms *Crithidia* and *Herpetomonas*, as in the case of *Proteosoma*, etc., will undoubtedly persist, but not as representing distinct genera. With the recognition of the trypanosomal nature, it does not follow that all of these flagellates are capable of growing in the blood of higher animals. Parasitism in the living blood must be looked upon as the result of a previous adaptation to the more or less digested blood in the intestinal tract of sanguivora. From this standpoint it will be seen that the ancestral, and for that matter the normal, type is the one met with in the digestive tube. Hence, the cultural forms, instead of being regarded as degenerations or involution types, in reality represent the normal phase, whereas the blood forms are transitory modifications due to environmental conditions.

METHODS EMPLOYED.

In order to study the flagellate infection of "wild" mosquitoes, the latter were captured in large numbers by means of Nocht's tubes. They were nearly all obtained from the thick undergrowth along the river bank in Ann Arbor; and, as soon as they were brought to the laboratory, they were placed in wire cages and allowed to feed on perfectly "clean" normal animals. By "clean" we mean animals which culturally and microscopically were shown to be free from trypanosomes and hemocytozoa. The mosquitoes were almost wholly of the genus *Culex*, and the most common species was *C. pipiens* or *pungens*. There were always a few *C. sylvestris* and *C. triseriatus;* the identification of these two we owe to Dr. L. O. Howard. The *Anopheles* were very scarce, only a few *A. maculipennis* and *A. punctipennis* being captured. For the percentage of infections see p. 242.

At first the insects were fed on young pigeons, but at times hawks, crows, rats, and guinea-pigs were made use of. In order to prevent the mosquitoes being killed, it is necessary, as a rule, to immobilize the animal. In the case of the pigeon this can be done by merely

tying the wings against the body. Hawks were managed best by placing them on a Latapie holder, but the crows were with difficulty prevented from eating up the mosquitoes. Contrary to the general statement that these insects will not feed on rats, we have found that they do so, though perhaps not so freely as on pigeons. On placing the neck of the rat in the pillory of a Latapie holder, the mosquitoes will feed undisturbed. In general, the guinea-pig is the animal of choice, for the reason that it is readily attacked by the mosquitoes, and, since it does not attempt to destroy them, there is no necessity to secure it in any way. Although by no means necessary, it is advisable to trim off some of the hair or feathers on the exposed animal.

Usually the exposure to the mosquitoes took place during the early part of the evening, but the number of insects which then feed is relatively small. By allowing the guinea-pig to remain in the cage all night, a much larger number will feed, especially during the early hours of the morning. Each cage contained a hundred or more mosquitoes, but the majority of these would not feed. After removing the animal from the cage, the insects which fed were taken up in Nocht's tubes and set aside in a cool, shaded place. The wide end of the tube was covered with netting, while the trap end held a plug of cotton, moistened with water. It is not advisable to moisten it with sugar-water unless the insects are to be kept alive for many days.

The mosquitoes which thus fed on normal blood were kept from 30 to 70 hours, when they were examined for flagellates. The time for making the examination depended somewhat upon the temperature, for in very warm weather complete digestion took place in about two and a half days, whereas when it was cool three or more days were needed. The percentage of findings was rather low when the mosquitoes were examined early—that is, when the stomach was still distended with undigested blood. Evidently the flagellates had not multiplied sufficiently to permeate the entire contents, and hence could be easily overlooked. Similarly, when the examination was delayed until complete digestion had taken place, or when sugar-water had been fed, the flagellates were equally difficult to find, owing to the fact that the free-swimming or monadian forms, as designated by Léger, had passed into the resting or gregarine stage. The best results were

always obtained when the examination was made toward the close of the digestion period—i. e., 40–60 hours after feeding.

In all of these experiments the mosquitoes were examined after a single feeding, and it is quite likely that the percentage of infections would have been even higher had they been fed two or three times so as to obtain an increased multiplication of the flagellates.

The mosquitoes were killed by inverting the tube over a filter paper on which was placed a drop of chloroform. This does not appreciably affect the flagellates, which remain alive and active in the stomach. After removing the thorax, wings, and legs, the digestive tube can be drawn out in the usual way, and the contents then forced out into sterile salt solution. Owing to the large number of examinations which had to be made, it was found that direct pressure on the dismembered body, by means of a sterile lance-shaped needle, gave about the same result, and this shorter procedure of getting at the stomach contents was the one chiefly used. By this means the fluid contents were expelled into a drop of sterile salt-citrate solution (a half per cent of each); the slide and cover-glass were previously flamed. The yellowish or whitish liquid was then examined with a No. 7 Leitz objective, and when necessary with an oil immersion lens.

When present, the flagellates are easily recognized by their more or less active motion. After a preliminary study of the organisms, the cover-glass was carefully drawn off, dried in the air, and stained by a modified Romanowsky method. When deemed advisable, the fluid remaining on the slide was taken up with a few drops of salt-citrate solution and either injected into birds or transferred to blood-agar tubes.

The flagellates present in the stomach contents were injected into 20 canaries, two mourning doves, and two blackbirds. The injections were made intrapleurally, and in no instance was it possible to observe the development of either trypanosomes or intracellular parasites in the blood of the birds thus inoculated. It may further be added that the pigeons, etc., after the mosquitoes fed on them were examined microscopically, and later cultures were made from their blood, but no evidence of infection was obtained.

The different cultures which were obtained were likewise injected into many animals such as sparrows, canaries, pigeons, doves,

hawks, owls, crows, rats, and mice. Inasmuch as such cultures were very rich in flagellates, the number injected was clearly many thousand times greater than would be introduced into an animal by the bite of a mosquito. The results were negative, as in the inoculations with the stomach contents. It may be that the failure to produce an infection was because of the use of non-susceptible animals. Some vertebrate may be capable of infection with the mosquito flagellates, but as yet nothing is known on that point.

One infection experiment is specially worth mentioning. Four sparrows which had been previously examined and found clean were injected with a second generation culture of Strain No. 85 (*Crithidia*). After eight days one of the sparrows showed halteridia, another showed halteridia and proteosoma, while the other two remained clean. Apparently, two out of four sparrows developed halteridia as the result of the injection and it will be noticed that the same percentage, three out of six, was obtained by the Sergents in their work on the owl halteridium. It is reasonably certain, however, in our experiment that neither the halteridium nor the proteosoma was derived from the injected material. We have repeatedly found sparrows which at first were apparently clean, but after being kept in the laboratory for a week or two developed a halteridian or proteosomal infection. Latent infection is a common occurrence in sparrows, and for that matter in other birds, and, as pointed out on page 231, this condition may account for the apparently successful infections obtained by Schaudinn and by the Sergents.

For the cultural work the blood agar was prepared in the usual way. Defibrinated rabbit's blood was employed, and this usually was added, in the proportion of one to two, to the melted agar, previously cooled to about 50°. The slanted tubes, when solidified, were inoculated, by means of a drawn-out tube pipette, with the citrate suspension of the stomach flagellates, after which they were capped and set aside at the room temperature.

The chief obstacle to the successful cultivation of the mosquito flagellates is the constant presence of bacteria in the intestinal canal of the insect. The bacteria, on account of their more rapid multiplication, may outgrow the flagellates from the very start, and thus render the medium unsuitable for the latter. This may be brought

about by a change in the reaction, or by the production of directly injurious products, or by the abstraction of oxygen. The abundant growth of aerobic bacteria may, as is well known, lessen the oxygen contents of a liquid to the extent of producing an anaerobic condition, which we know would interfere with the growth of trypanosomes in the culture-tube. In the stomach of the insect for some reason (possibly the reaction) the bacteria do not develop readily, and hence the trypanosomes are able to multiply, at times, to a very marked extent.

As might be expected, the flora of the insect gut is subject to considerable variation, and when slow-growing yeasts or bacteria predominate, the chances of getting the trypanosomes to grow, at least for one or two generations, are favorable. The conditions are seemingly more favorable when only a single species of bacterium or yeast is present. Our most successful results were obtained with such "pure mixed" cultures. The isolation of the trypanosomes from the accompanying bacteria or yeasts offers some difficulty, but this we were able to overcome, as will be shown later on, by plating on blood agar.

Cultures were attempted from 89 mosquitoes, all of which contained trypanosomes, some even in very large numbers. Of this number only seven were positive, Strains 10, 11, 18, 29, 69, 85, and A. Strains A and 69 were crowded out by bacteria after two and three days respectively. Strains 18 and 85 were kept alive for 20 days, through two and five generations respectively. Strain 11 was carried in 30 days though four, and Strain 29 in 166 days through 24 generations. Strain 10, which was started on August 5, 1905, is now (April 1907) in its 87th sub-culture.

Of the two cultures which were kept up one, Strain 10, was *Crithidia fasciculata* and the other, Strain 29, was a *Herpetomonas*, *Tr. culicis*. The former was associated with a yeast and the latter with a minute bacillus. The growth was very rapid and extremely rich, so much so that in warm weather it was necessary to check it by keeping them in a cold room. These two cultures will be described in their proper place.

CRITHIDIA FASCICULATA.

This flagellate is fairly common in the gut of ordinary "wild" mosquitoes of the genus *Culex*, but it is not the only organism that

may be present. Thus, out of 882 mosquitoes examined in 1905, flagellate infection was met with in 126 (14.3 per cent). Of this number, 55 had apparently only *Crithidia* (6.2 per cent), while 52 had only *Herpetomonas* (6.0 per cent), and 19 had both parasites (2.1 per cent). During the summer of 1906 the percentage of infected mosquitoes was about a third of that found in the preceding year. Thus, of 746 mosquitoes 37 were infected (4.95 per cent). Of this number 20 had only *Crithidia*, 16 only *Herpetomonas*, and one had a double infection. It will be seen from this that infection with the former is slightly more common than with the latter. The relatively small number of infections obtained last year was probably due to the cutting-away of most of the undergrowth along the river.

The existence of a mixed infection with these two flagellates has not heretofore been recognized, and as a result, the two types, when occurring together, have been described as different stages of one and the same organism. This is clearly the case with the *Herpetomonas subulata* of Léger, for the small pear-shaped, truncated forms which he shows (Ref. 35, p. 614, Figs. 4 and 5) are *Crithidia*, while the longer ones (Figs, 1, 2, and 3) are true herpetomonads. Again, the *Crithidia minuta* which Léger, on account of the presence of the long form (Ref. 35, Fig. 6, p. 614; also Ref. 33, Fig. 3, p. 185) later placed in the genus *Herpetomonas*, is without doubt a true *Crithidia*, since it shows the short pear-shaped as well as the elongated, cylindrical form which belongs to this type. These two figures of Léger should be compared with Fig. 6, Plate 8. Our reason for this belief is based upon the results of the cultivation of the two types, for it will be shown that in cultures the *Crithidia* and *Herpetomonas* present entirely distinct forms and retain their individual characteristics through numerous sub-cultures. In cultures of *Crithidia* the typical long herpetomonad form, gradually tapering posteriorly, is never found, and similarly in cultures of the *Herpetomonas* there is an entire absence of the peculiar short crithidian type.

On account of the facts just stated we cannot agree with Lühe in his characterization of the genus *Crithidia*. According to Prowazek, the *Herpetomonas* of the house-fly has a double flagellum arising from a terminal blepharoplast, but this observation, as already pointed out, needs confirmation. The absence of the double flagellum,

the position of the centrosome at some distance from the anterior end, and the presence of a feebly developed undulating membrane are regarded by Lühe as the most important generic features of *Crithidia*. These properties, however, are based presumably upon the long form of *H. subulata*, which is entirely different from that of true *Crithidia*. Hence, until it is definitely shown that the *Herpetomonas* of the house-fly always possesses a double flagellum, it will be well to retain this designation for similar forms found in other insects.

The genus Crithidia, *if it is to be retained, is characterized by peculiar, short, oval or pyriform bodies which are usually rounded or obtuse posteriorly, while the anterior end is truncated, or even slightly depressed, and bears a short, straight flagellum. In this short form the nucleus is near the posterior end, and the blepharoplast is by its side. Somewhat longer cylindrical forms, tapering very slightly, or rounded at each end, may be present, and these are provided with a long flagellum. In this form the nucleus is near the center, and the centrosome is usually about midway between the nucleus and the anterior end. Both forms may occur in rosettes with flagella directed centrally. The undulating membrane and the posterior diplosome, as seen in the* Herpetomonas, *are absent.*

Some variation in the form and size of the *Crithidia* is met with in the body of the mosquito. This must obviously be the case in view of the changes in the composition and concentration of the intestinal contents. It will be sufficient to point out the great difference in the rate of digestion of the blood in the stomachs of mosquitoes according as they are kept at a high or a low temperature. Furthermore, the ingestion of plain water or of sugar-water is an additional factor in bringing about greater or less alteration in form and size. In the cultures on blood agar these differences are readily observed, as will be presently shown. The environmental conditions are essentially the determining cause of the two types of Léger—the free or monadian, and the fixed or gregarine-like, resting stage.

The possibility of the existence of several species of *Crithidia* is obvious, and it is likely that their differentiation, as in the case of bird trypanosomes, can be effected more readily by the cultivation method than by mere morphological study. In the course of this investigation we have grown five strains of *Crithidia*—Nos. 10, 11,

69, 85 and A; but unfortunately all but the first were soon lost. It was noticeable, however, that Strain No. 85 differed in some respects from the others, but this variation might have been due to changes in the medium caused by the different kinds of bacteria present. What has been said here with reference to the existence of several species of *Crithidia* is also true for the *Herpetomonas*. The question is worth investigating in localities where flagellate infection of mosquitoes is very common. Taken as whole, the *Crithidia* which we have studied, in mosquitoes and in cultures, agree so well with the description of *C. fasciculata* which Léger found in *Anopheles* that we have identified all of our forms with this species.

MOSQUITO FORMS.

The flagellates may be present in the stomach contents in varying numbers. In some instances but one or two may be found on the slide, while in others they are exceedingly numerous. They may be single or in large masses which fill the field of a No. 7 objective. For convenience the *Crithidia* will be divided into the short and the long forms.

As seen in the living condition the *Crithidia* usually appear as short, thick, oblong cells. They are more or less actively motile and travel about with the whip foremost. The body of the smaller forms is about 3–4 μ long and 2 μ wide,while the larger ones measure 6–8 μ in length by about 3 μ in width. The contents are colorless and apparently homogeneous.

The short forms are often found grouped in rosettes with the whips inside. These groups may consist of large numbers of cells, just as in the case of the rosettes of *Tr. Lewisi*, *Tr. avium*, etc. The short form, furthermore, may become attached by means of the flagellum to the intestinal wall, more especially to the lower end of the Malpighian tubules, and as a result the entire wall may be covered with radiating masses consisting of countless numbers of parasites. A similar arrangement of the cells is to be seen about minute globules of air (aerotropism), and this behavior so characteristic of cultural trypanosomes will be discussed later on.

The most important features are brought out in the stained preparations. The thick wide cells may be said to be pear- or cigar-

shaped (Plate 7) and have been compared by Léger to barley grains. The posterior end is either round or slightly pointed. The body of the cell may, or may not, taper toward the anterior end, which is either square or slightly concave. Round forms, from 4 to 6 μ in diameter, may also be present. The relatively large round nucleus lies near the posterior end in the short forms and at about the middle in the longer ones; close to the nucleus in front or on the side is the centrosome. From the achromic zone surrounding the latter, the flagellum can be seen to pass forward and out *at the middle* of the anterior end. The free portion of the whip is short and straight, or but slightly bent. The flagellum is more difficult to stain than in the case of other trypanosomes, and hence the cells will often appear to be devoid of whips. A colorless channel, marking the site of the flagellum, can be seen in such specimens, extending from the blepharoplast to the anterior end (Figs. 1 and 2, Plate 8). In the stained preparations the body of the cells measures from 3 to 8 μ in length and from 1.5 to 3 μ in width. As the free whip is from 2 to 5 μ long, the total length of the cell may be put down at from 5 to 14 μ. These measurements, it may be said, have been made on preparations made from mosquitoes which contained no herpetomonad forms. The absence of very long forms should be noted.

CULTURAL FORMS.

As mentioned on p. 241, one of the crithidian strains which were isolated from mosquitoes (No. 10) has been cultivated now for 20 months, and during that time has passed through 87 generations or sub-cultures. For more than a year it has been grown as a strictly pure culture.

On the ordinary blood-agar medium it grows even more rapidly than any of our bird trypanosomes, so much so that during the warm weather it is necessary to restrain the growth by placing the tubes in a cool room. Without this precaution it would be necessary to transplant the culture every three or four days. The growth is extremely rich, and glairy patches or colonies are readily made out. Obviously, depending on the changing conditions of the medium and temperature, some variation in form and size will occur, but on the

whole the cultural features of our strain have been maintained unimpaired during these many months.[1]

It is important to note that these cultural forms are very often of the same size and shape as are those found in the mosquito. This fact will be readily seen on comparison of the Plates 7, 8, and 9. As in the gut, so in the tube we can distinguish between a very short and a moderately long form. The short thick cells (body 4 to 8 μ long and 2 to 3 μ long) with truncated anterior are identical with like forms seen *in vivo*, the gregarine forms of Léger. On the whole, the conditions in the test-tube, notably the abundant food-supply and absence of bacteria and other inhibiting influences, are more favorable than in the insect, and there is a greater tendency to develop the long or monadian form. This is entirely different from that of the *Herpetomonas*, and, as will be seen, it is much shorter than the latter. The body of the long form measures from 8 to 15 μ in length and from 1.5 to 2 μ in width. The free flagellum may be as long as the body, and hence the total length of the cells may range from 13 to 26 μ. The width of some of the forms, especially just before division, may reach 4.5 μ. These long cultural forms are represented by similiar ones (10–15 μ in length), resembling *Herpetomonas*, which are found at times in the mosquito. The cylindrical or cigar-shape, the obtuse posterior end, and the very short taper to the anterior end of body, together with the absence of the posterior diplosome, makes it possible to distinguish the elongated *Crithidia* from the true *Herpetomonas*. (See p. 254.)

It is important to note that the long cultural forms, at times, show a marked constriction near the posterior end (Figs. 4 and 5, Plate 9). Although we have not ascertained the real significance of this constriction, we are inclined to consider it as a process preparatory to actual division. This feature corresponds, in a remarkable way, with the constriction of the oökinetes of *Tr. noctuae* as described by Schaudinn (see his Figs. 1, 2, and 3).

While this fact, together with the rounded posterior end, goes to establish a certain identity between *Tr. noctuae* and *Crithidia*, it may be well to point out that the former does not agree in all respects

[1] At the present time the vegetative power of this organism is such that it can be grown on ordinary inclined agar without the addition of blood. In several attempts to secure "blood" forms, by resorting to cultivation in collodium sacs in rats, no growths were obtained.

with the latter. This is especially true of the anterior end, which in *Tr. noctuae* is prolonged along the base of the flagellum and clearly shows an undulating membrane. These two characteristics, and the presence of posterior diplosomes, agree with the structure of *Herpetomonas*. It would seem therefore as if Schaudinn's *Tr. noctuae* was a composite of these two flagellate forms.

Rosette formation is a common feature of the cultures and corresponds to the characteristic grouping met with in insects. The smaller groups of from two to 10 cells usually consist of short thick ovals or spherical bodies which, as in the case of the rosettes of *Tr. avium, Tr. Lewisi*, etc., show little or no motion. At times the rosettes consist of many hundreds of individuals, which may be partly or wholly of the elongated type, and in such cases the latter show an active swaying motion. The flagella are directed centrally as in the case of all multiplication rosettes.

The rosettes are easily distinguished from ordinary agglutinations. The latter result in irregular masses, consisting at times of numerous cells, all of which have the flagella on the outside. The beginning of agglutination may be seen when two cells become adherent by their posterior ends; or side to side with the flagella extending in opposite directions.

Another very important feature which seemingly is shared by all cultural trypanosomes, but not by the blood forms, is the phenomenon of *aerotropism*. Thus, if a minute bubble of air is introduced under the cover-glass, the *Crithidia* will range themselves in compact rows, two to five deep, around the globule. Every cell has its whip directed toward the air-bubble. This behavior of *Crithidia*, and of cultural trypanosomes in general, to air corresponds, as will be seen, to the so-called gregarine or resting stage observed in the insect. In the latter the flagellates are attached in the same manner to the epithelial cells, and it is reasonable to believe that this so-called resting stage is in reality an aerotropic manifestation due to the alteration and absorption of the stomach contents. Certain it is that this resting form when detached from the epithelium, by pressure or currents of liquid, is at once able to move about quite freely.

The cultural fluid, in addition to the rosettes, shows many free, actively moving, darting forms. These may travel through the field

with great rapidity and in nearly straight lines. Most of the single forms, however, for some reason become attached by means of their flagella to the glass surface and are unable to do more than sway or wriggle about the attached point.

The length of the flagella varies with that of the cell, and in general is about as long as the body proper. In the small forms the whip is short and stiff, while in the elongated type it is long and flexible. In the former it is not unusual to see the flagellum retracted to a short stiff bristle as a result of the passage of the blepharoplast from the anterior end of the cell to the side of the nucleus which lies at or near the further end. (See Figs. 1, 2, and 3, Plate 9.)

The larger *Crithidia* show a distinct yellowish green color, which may be due to staining with blood pigment. The contents of the cell appear homogeneous, or show at most a very fine granulation. In heavily stained preparations deeply colored granules can be seen filling the cell (Plates 8 and 9). This stippling is at times difficult to bring out. In Strain 85 the cultural cells were totally colorless and showed very minute but clear, distinct globules.

The presence of an undulating membrane has not been satisfactorily established. The short, oblong forms, on account of the square anterior end and median insertion of the flagellum, are clearly devoid of an undulating membrane. In the very longest forms the anterior end is not carried along the flagellum, as in the case of *Herpetomonas* and most trypanosomes, but is seen to terminate more or less abruptly. Such long forms, when examined in the living condition, especially when the cell has become sluggish, give evidence of the presence of an undulating membrane at the base of the flagellum. The periplast is apparently carried beyond the obtuse or truncated end of the cell as a short triangular membrane which shows a *distinct wave motion*. With the retraction of the flagellum, as in the short form, all evidence of this structure disappears. In the stained preparations it has not been possible to demonstrate this rudimentary membrane, although indications of it are to be seen in Fig. 6, Plate 8.

The presence of an undulating membrane, imperfect as it is, definitely places the *Crithidia* among the *trypanosomes*, and this position is further indicated by the obvious analogies which this type presents in cultures to those of the latter.

The very small rounded forms, especially when the flagellum does not stain, resemble very much, as Léger first pointed out, the *Piroplasma*. The resemblance to the peculiar parasite of kala-azar, *Piroplasma donovani*, is even more marked. The development of the latter into flagellate forms in the test-tube (Rogers), and in the bedbug (Patton) suggested to the former a relationship to *Herpetomonas*, while the latter is of the opinion that this parasite is a *Crithidia*.

Divisional forms, of course, are very common in the cultures, and hence such material presents the very best opportunity for the study of the life-history of the parasite. The forms met with are the same as those seen in the mosquito. The short, wide ovals divide longitudinally into two equal halves, each of which shows the typical square anterior end (Fig. 3, Plate 8).

As mentioned on p. 239, the inoculations of birds and mammals with large amounts of the flagellates, obtained from tube and flask cultures, failed to produce an infection. Hence, so far as we know, the *Crithidia* is a parasite peculiar to the mosquito, and perhaps to other insects.

TRYPANOSOMA (HERPETOMONAS) CULICIS, N. SP.

It has already been shown that the ordinary "wild" mosquitoes of the genus *Culex* may harbor either *Crithidia* or *Herpetomonas*, or both kinds of parasites at the same time. From the figures given on p. 242 it will be seen that the infection with *Herpetomonas* is nearly as common as that with *Crithidia*.

The *Herpetomonas* which we have studied resembles in general the long monadian form of *H. subulata* which Léger found in *Tabanus glaucopis*, and at first we were inclined to identify it with the latter. Léger's form, however, is distinctly longer, the body on an average being 30 μ long by 1.5–2.0 μ wide, the flagellum being 20–25 μ long. Moreover, as pointed out on p. 242, the description of this species undoubtedly includes a true *Crithidia*. The *Herpetomonas algeriense* which the Sergents described, shortly after the appearance of our preliminary note, was found in 13 specimens of *Culex pipiens* and in one of *Stegomyia fasciata*, and was characterized as having the centrosome in the motile forms, posterior to the nucleus, and also by a rounded resting form having very long flagellum. The unnamed type

which they found in the digestive tube of a larva of *Anopheles maculipennis* resembles our form more closely than does the preceding. It appears that, notwithstanding its frequent occurrence, this mosquito flagellate has not been heretofore recognized, or at all events, it has not been differentiated from the accompanying *Crithidia*. We therefore consider it as a new species and propose to designate it as *Trypanosoma culicis*.

While this organism has been found only in the several *Culices* examined by us, it is quite probable that it will be recognized in other mosquitoes, including *Anopheles* and *Stegomyia*. It is hardly necessary to add that a further investigation of the mosquito flagellates is needed in order to pass upon the question of the multiplicity of species both of *Crithidia* and *Herpetomonas*. The wide geographic distribution of these two types is seen in the fact that Patton has found both of them to be very common in the *Culex fatigans* at Madras. The probable presence of the two types or genera in other insects is evident from Léger's work on the Tabanides.

According to the recent studies of Prowazek, the herpetomonads of the common house-fly and of the meat-fly (*H. muscae domesticae* and *H. sarcophagae*) possess a double flagellum which terminates in a minute diplosome, and this, in turn, connects with a single large blepharoplast by means of two rods or rhizoplasts. As pointed out, if these observations are confirmed, it will be necessary to restrict the term *Herpetomonas* to those organisms having the same flagellar arrangement as in the type species *H. muscae domesticae*. It seems to us, however, somewhat premature to insist, as Lühe has done, upon the limitation of this genus to these two double flagellar forms. The herpetomonadian forms have been noted in diverse insects, but in none has this flagellar structure been seen. Such an arrangement is certainly absent in the herpetomonadian forms which we have studied, namely *Tr. culicis* and *Tr. Grayi*.

On the other hand, there are reasons for believing that the double flagellar arrangement described by Prowazek represents an early divisional stage. Such undoubtedly is the case with the forms described in the *Stomoxys* by Gray. It is to be remembered that the first evidence of division is usually seen in the enlargement of the blepharoplast, and then in the formation of a new flagellum (see

Fig. 3, Plate 11). As a matter of fact, in rapid multiplication, such as is seen in the segmenting forms of *Tr. Lewisi*, the cultural forms of *Tr. Laverani* (Ref. 46, Fig. 6, Plate 6), etc., the new whip appears before the blepharoplast shows any appreciable sign of division. The existence of a double flagellum would effectually remove the flagellates of the house-fly from the trypanosomes. The *Herpetomonas* of the mosquito, which it will be seen is a true "cultural" trypanosome, shows considerable similarity to *H. muscae*.

MOSQUITO FORMS.

What has been said on p. 244 with reference to the variation in the form and size of *Crithidia*, and the possible existence of several species, holds equally true for the *Herpetomonas*. The conditions which obtain in any two insects are not necessarily alike, and, for that reason, considerable difference in size and form may be expected without recourse to the assumption that very long forms represent one species and very short ones another. The variation in size is an important argument in support of the view that the intestinal flagellates are true cultural types.

In the living condition the *Herpetomonas* is very active and moves about rapidly; so much so that it is often difficult to follow it from field to field. It moves with the long flagellum foremost in exactly the same manner as do the cultural trypanosomes, *Tr. Lewisi*, *Tr. avium*, etc. The contents of the cell appear colorless and homogeneous.

On account of the rapid motion the form is at times difficult to make out, but in cells which have slowed down, and especially in stained preparations, it will be seen that the cell is a narrow spindle tapering gradually toward each end. The presence of an undulating membrane is clearly indicated in the longer forms.

The herpetomonad forms seen in the living preparations vary considerably in length. In a few mosquitoes very long forms, the body measuring from 30 to 35 μ, have been observed. In these the flagellates were all of this size, and, had the cultivation attempts been successful, it would have been possible, perhaps, to determine whether this long form was a distinct species. In the majority of the mosquitoes the herpetomonad forms were from 12

to 20 μ long. The width of the living cell is more uniform and varies from 0.5 to 1.0 μ. Hence, the appearance of the long, slender form is not unlike that of the spirochete-like stage of *Tr. avium*. It is a mistake, however, to compare either of these types with the true spirochetes, inasmuch as there is an entire absence of the spiral winding so characteristic of the latter and, moreover, on staining, there is brought out the usual trypanosomal structure which is wholly lacking in the real spirochetes (*Sp. Obermeieri*, *Sp. Duttoni*, etc.).

The presence of these very long, slender forms in the mosquito, however, is very strongly suggestive of Schaudinn's *Spirochaeta Ziemanni*. It will be remembered that Schaudinn himself practically acknowledged that this organism was not a true spirochete, since it had the ordinary trypanosomal structure. In view of this similarity, it may well be asked whether or not some of the forms of this *Herpetomonas* correspond with the flagellate stage of *Hemoproteus Ziemanni* as described by Schaudinn. Certain it is that the long, slender forms correspond to the size (25 by 1.0 μ) given by the Sergents for the flagellate stage of *H. Ziemanni*. On the other hand, it should be noted that Schaudinn's Fig. 17 shows a long undulating membrane, due to the position of the blepharoplast which is posterior to the nucleus, as in the cultural form of *Tr. avium*. For the cultivation of spiochetes from owls see p. 259.

In addition to the long and short forms mentioned, various stages of involution are met with as in the case of ordinary cultures. The more or less spherical forms with long flagella, kite-shaped bodies and spindles, rounding up posteriorly, correspond to the resting stage of Léger.

Divisional forms are often seen in the living preparations. The dividing cells are shorter and wider than the ordinary free-swimming forms. The division is longitudinal and is fairly equal. Multiplication rosettes with centrally directed whips are occasionally found, but they are by no means so common as in the culture tube. Agglutination groups or rosettes have also been observed, and it is important to note that in these groups the flagella are directed outward as in the case of *Sp. Ziemanni*.

The herpetomonad forms found in the stained preparations

made from mosquitoes vary greatly in length, from 10 to 45 μ. They may for convenience, if for no other reason, be divided into four types: (1) long; (2) medium; (3) short; and (4) wide.

1. In the long spindles the posterior two-thirds of the body gradually tapers to a fine point, while the anterior third is drawn out along the base of the flagellum so that it is difficult to tell just where it ends (Fig. 2, Plate 10). The length of the body in these long forms varies from 25 to 35 μ, and the greatest width is from 1.0 to 1.5 μ. The free whip measures from 5 to 10 μ, so that the total length ranges from 30 to 45 μ. *With respect to size, as pointed out above, this type corresponds to the forms which the Sergents identified with the flagellate stage of* H. Ziemanni. (See p. 231.)

2. The cells of medium size,[1] those from 15 to 25 μ in length (not including the whip), likewise taper toward both ends. The width may be a trifle greater than that given for the preceding type—that is, from 1.5 to 2.5 μ (Figs. 1, 2, 3, Plate 10). *This type corresponds in size to the indifferent forms of* Tr. noctuae *as given by the Sergents.*

3. In the short form the body measures from 7 to 15 μ, and the whip is from 3 to 8 μ long. The width is about the same as that of the preceding. These short forms also show the characteristic tapering ends, though not to the same extent as in the long types (Fig. 6, Plate 10). *They may be compared with the male forms of* Tr. noctuae *as given by the Sergents* (p. 227).

4. In addition to the three types given above, which are by far the most common, there is another present, in mosquitoes and in cultures, though it is much less frequent. This is a wide, fairly long form in which the characteristic tapering is less pronounced (Figs. 5 and 6, Plate 12). The body measures about 20 μ in length and about 2.5–3 μ in width. The free whip is short, about 5–8 μ. This type stains a deeper blue than any of the above, and the contents show granules as well as colorless globules. *This form may be identified with the female form of* Tr. noctuae *as described by the Sergents* (see p. 227). It recalls the broad or "female" type of *Tr. Lewisi* as met with in the blood of rats, and may well be compared with the broad or "female" type of *Tr. Grayi* (Ref. 45, Plate 15).

[1] The long and median narrow forms described above are suggestive of the slender or "male" type of *Tr. Grayi*, as will be seen on comparison of Figs. 1 and 2, Plate 10, with Figs. 1 and 2, Plate 15 (Ref. 45).

Inasmuch as all four of these types have been studied in the cultures of *Tr. culicis* as well as in the mosquito, there is every reason to regard them as belonging to one species. Similar variation in form and size, it may be added, is common among other cultural trypanosomes, for example, *Tr. avium.*

The nucleus in the narrow forms is compact and cylindrical, and as wide as the cell (compare *Tr. Grayi*, Ref. 45, Figs. 1, 2, and Plate 15). In the "female" type two nuclei may be present without there being any evidence of multiplication (Fig. 5, Plate 12). Similar polynuclear forms have been described in the cultural forms of *Tr. Grayi*, *Tr. Lewisi*, etc.

The blepharoplast is round or oval and measures 0.5–0.7 μ. It lies either immediately in front of the nucleus or about midway between the latter and the anterior end. We have never seen it lie posterior to the nucleus as in *H. algeriense.* Starting from the blepharoplast, a single flagellum passes forward along the side of body and is finally prolonged as the free whip. In the long forms the evidence of the presence of an undulating membrane is unmistakable (Fig. 2, Plate 10).

By far the most characteristic feature is the presence of a *posterior diplosome* or *acrosome* similar to that which we first described in the cultural form of *Tr. Laverani* (Ref. 46, p. 290, Plate 6), and in *Tr. Grayi* (Ref. 45, p. 407, Plate 16). This body is present in all four types as well as in the dividing forms. It appears to be a definite, constant structure, as much so as the nucleus or the blepharoplast. The presence of this diplosome has not been observed in *Crithidia.*

As a rule, the diplosome stains less readily than does the nucleus, and this behavior is probably due to the protecting action of the surrounding matter. In the very thin, or in crushed, cells this structure is as sharply defined as the blepharoplast (Fig. 6, Plate 11). The diplosome is usually rod-shaped, about 0.4 by 1.6 μ, with a more or less evident median constriction, in which case it presents the appearance of a diplococcus or diplo-bacillus (Fig. 1, Plate 10). It is nearly as large as the blepharoplast.

The diplosome apparently divides before any divisional change is seen in the nucleus or blepharoplast (Figs. 4 and 5, Plate 10). As

the result of such division two distinct rods or two diplococcus-like bodies form. We have never seen more than two diplosomes in a cell, although special search was made. A suggestion of three such bodies may be seen in Fig. 5, Plate 10. In the first three types the diplosome is always posterior to the nucleus; in the fourth or "female" type it may lie in the anterior part of the cell.

A somewhat similar structure, derived from the blepharoplast, has been described by Schaudinn in connection with the development of the oökinete into the female and male forms of *Tr. noctuae* (see his Figs. 2 and 4). In that case the blepharoplast is said to give rise by three consecutive divisions to eight small, compact nuclei, each of which in turn divides, forming a smaller nuclear body. In the female cell, these eight pairs of nuclear bodies disappear, whereas in the male oökinete they become the nuclei and blepharoplasts of eight male trypanosomes. In his study of the *Herpetomonas*, Prowazek has likewise noted a posterior diplosome which is apparently in relation to the blepharoplast.

The diplosome which we have described is present in all forms of *Tr. culicis*, as found in the mosquito as well as in those grown in the culture-tube. The similar bodies of Schaudinn, it should be said were noted only in the oökinete. Just what the significance of the diplosome may be it is impossible to state, but our observations go to show that it is a distinct morphological structure and is not directly derived from the nucleus or blepharoplast. As pointed out above, the diplosome presents the earliest evidence of a coming division of the cell. As the latter divides, each half is equipped with a diplosome (Figs. 2 and 6, Plate 11), as in the case of *Tr. Laverani.*

The direct division of the long, slender forms of *Tr. culicis* have not been observed. They apparently shorten and widen just before division takes place. At all events, the common dividing form, as found in the mosquito and in the culture, is an oval body about 3 μ wide and 8–10 μ long. The acrosome divides first; then the blepharoplast, which lies just in front of the nucleus, divides and gives rise to a new flagellum, after which the nucleus divides (Fig. 3, Plate 11). The division is equal and longitudinal, and hence the resulting two cells are of about the same size about 8–10 μ long and 1.0 μ wide (Fig. 6, Plate 11).

The female type apparently divides longitudinally without becoming round or oval (Fig. 6, Plate 12). The two large nuclei which result from division separate, and one lies in the anterior and the other in the posterior part of the cell. Hence, one of the cells resulting from the division will have its nucleus at or very near the posterior end. In this dividing form the diplosomes lie either between or in front of the nuclei (Fig. 5, Plate 12), as in the case of *Tr. Grayi.*

Involutions.—In some mosquitoes the *Herpetomonas* loses its typical form, rounds up posteriorly, and becomes first club-shaped, and finally more or less spherical. This change likewise occurs in the culture-tube, and the resulting bodies correspond to the round form seen in cultures of *Tr. Lewisi, Tr. avium*, etc. Such bodies, measuring about 5 μ in diameter or 5 by 8 μ, represent in part the resting or gregarine form of Léger and others, and should probably be interpreted as degenerations or involutions, since the plasma in these altered cells does not stain as well as in normal cells. In such cells, owing to the feebly stained protoplasm, the nucleus, blepharoplast, and diplosome stand out very prominently (Figs. 3, and 4, Plate 12). As opposed to the view that these are degenerations is the fact that these forms show the various division changes. Thus, one may find all stages from the cell with a single nucleus, blepharoplast, diplosome, and whip to one in which each of these structures is doubled.

CULTURAL FORMS.

Of the seven cultures of mosquito flagellates only one (Strain No. 29) gave a good growth of *Herpetomonas* which was successfully carried through 24 generations or sub-cultures in the course of five months, from August 6, 1905, to January 12, 1906. Another, Strain No. 18, went through two passages, but was then lost on account of overgrowth by the accompanying bacteria. The cultures made from Mosquitoes 10 and 11 which had a double infection showed some herpetomonad as well as crithidian forms (in the first generation), but for some reason the former disappeared on transplantation, and the second and subsequent generations showed only *Crithidia.* The disappearance of *Herpetomonas* from these cultures indicates some

unfavorable action either of the particular kind of bacteria which were present or of the *Crithidia*.

Strain No. 29 was associated with a minute diplo-bacillus which seemingly exerted no injurious action as long as the cultures were transplanted regularly, once or twice a week. When the transplantation was delayed for a longer interval, most of the trypanosomes, as is usually the case, became degenerated, and hence the sub-cultures then made were poor in flagellates and relatively rich in bacteria. Otherwise there was no reason why this culture could not have been kept up almost indefinitely as in the case of *Crithidia*.

The isolation of a strictly pure culture was more difficult than with *Crithidia* on account of the adherence of the minute bacillus to the sticky surface of the flagellate. Several times, however, almost pure cultures were obtained from colonies on blood-agar plates, and, without doubt, this method on further replating would have given a strictly pure growth. Unfortunately, just at this time the culture was lost through an oversight in not making regular transplants. The streak cultures on rabbit blood agar, in a Petri dish, gave moist, glairy colonies of almost pure trypanosomes. The colonies varied in size from mere points up to that of a large pin-head.

The forms met with in the culture-tube, it may be said, correspond in every respect to those observed in the mosquitoes. The four types found *in vivo* are also present *in vitro*, and in size and form the latter are not to be distinguished from the former. This fact accords with the observations on the crithidian cultures, and confirms the view that the flagellates of mosquitoes and other insects represent cultural forms.

Nothing is known as yet regarding the forms which these flagellates would assume if they were able to grow in the blood current. It is probable, however, that they would give rise to large typical trypanosomes corresponding to the blood forms of *Tr. avium*, *Tr. Lewisi*, etc. It is evident, therefore, that the cultural forms, instead of being degenerations and involutions as suggested by some, really represent the ancestral primitive type from which the peculiar blood forms have evolved as a result of adaptation to the living fluids of the body. From the cultural stand-point the "drawn" blood does not

correspond to the living blood, by which term we may designate that which is circulating in the body.

The cultures of *Tr. culicis* show small forms, the body of which measures but 6 or 7 μ. These may have a tapering posterior end, or one which is blunt and rounded. They are readily distinguished from the wider and truncated *Crithidia*. Small, rounded or oval forms, 4 or 5 μ in diameter, are also present, and such usually have a very long flagellum, 12–16 μ in length. The most common form is that of the typical *Herpetomonas* with slender, tapering posterior end. These are from about 12 to 15 μ in length, and from 1 to 1.5 μ in width. Occasionally longer forms up to 35–40 μ, and the "female" forms already mentioned, are met with (Plate 12).

Rosettes, with the flagella directed centrally, are present in the cultures, but not as commonly as in the case of *Crithidia*. Agglutination of two or more cells is a common condition. The two cells may be attached either by their posterior ends or lengthwise, side by side, with the flagella pointing in opposite directions. When agglutinated in large masses the cells are also grouped with the flagella on the periphery. As with other flagellates the presence of soft débris or fat globules favors the formation of large agglutinations.

Aerotropism is as pronounced as in the case of *Crithidia* and other cultural trypanosomes. The long, slender herpetomonads arrange themselves around the globule in dense layers, side by side, with the flagella inward, and thus present a very striking object.

The slender forms travel rapidly, with the whip foremost, and while in motion appear as mere lines. The body is straight or slightly curved, and is rather stiff—a condition especially noticeable when the motion has decreased. The contents of the living cell have a greenish tint and homogeneous, but on careful examination very fine granules can be seen, especially in the posterior half. The presence of a slight, but distinct, undulating membrane can be observed in the slowly moving, or arrested long forms. Short, wide dividing forms (6–8 μ long by 4–5 μ wide) corresponding to those observed in the mosquito are fairly common (Plate 11).

Inoculations of the cultures were made into a variety of birds and mammals, as stated on p. 239, but in no case was it possible to secure an infection showing either trypanosomes or cytozoa. While it is

possible that this flagellate has a blood stage in some vertebrate, there is as yet no evidence of that condition, and, for the present at least, the *Tr. culicis* must be regarded as a parasite peculiar to the mosquito.

SPIROCHETES.

The so-called *Spirochaeta Ziemanni* which Schaudinn described in *Culex pipiens*, as the flagellate stage of *Haemoproteus Ziemanni*, is clearly a trypanosome and not a true spirochete. In view of this fact, it is of interest to note that Töpfer, according to Mühlens and Hartmann, has succeeded in cultivating a spirochete from the owl. Although Töpfer has not published as yet his results, we are assured by Dr. Schilling that the organism mentioned is a real spirochete, and, that being the case, it throws much light upon the exact nature of "*Spirochaeta*" *Ziemanni*, which Schaudinn himself practically acknowledged to be a trypanosome. Töpfer's observation would go to show the existence of a spirochete infection of owls similar to that known in chickens and geese, bats, etc. It is possible that mosquitoes feeding on owls, thus infected, would show true spirochetes in the digestive tube.[1]

[1] Since the above was written one of us (Novy), through the very great courtesy of Dr. Töpfer, has been able to examine a preparation and a photogram of the spirochete which he has cultivated from the blood of an owl infected with *H. Ziemanni*. There is no question as to the spirochetal nature of the organism which Dr. Töpfer has isolated. It is an interesting and important fact that this organism presents in cultures not only typical spirochetes 10–15 μ in length, but also smaller forms consisting of but one or two turns. The latter correspond to the S-, or vibrio, forms of the cholera spirillum. The cultures also show minute round bodies which contain one or two chromatin granules and these bodies doubtless represent the resting stage of *Sp. Ziemanni* as described by Schaudinn. They may be compared with the well-known involution forms of the cholera vibrio, and their presence, together with the short forms mentioned above, affords good evidence of the bacterial nature of this spirochete.

Dr. Töpfer's organism in all probability corresponds to the *Spirochaeta Ziemanni* of Schaudinn. The long spirochetes, and the short forms representing the minute ones found by Schaudinn in the gut of mosquitoes (the result of the rapid multiplication of the indifferent form), and also the round or resting forms, are in accord with Schaudinn's description. It appears probable, therefore, that the *Spirochaeta Ziemanni* after all is a spirochete and not a trypanosome. But assuming that this identity is established it does not go to prove that the spirochete in question is a stage in the development of the intracellular parasite *H. Ziemanni*. On the contrary, it indicates the existence of a spirochetal disease of owls corresponding to that of geese and chickens. Indeed, Schaudinn himself noted that the owls, inoculated with suspensions of mosquitoes, showed an enormous increase of indifferent spirochetes very much like that seen in infections with *Sp. anserinum*. He considered this as the acute stage which was later followed by the appearance of the sexual forms of *H. Ziemanni*. It may be said, as a result of our own observations, than an infection with *Sp. Obermeieri* or *Sp. Duttoni* seems to exert a marked favoring action upon the multiplication of trypanosomes and probably of other protozoa, and hence the change from one type to the other, as noted by Schaudinn, is suggestive of a double infection. It will be seen, therefore, that the owls are subject to infection with at least four parasites, namely *H Ziemanni*, *Halteridium*, *Trypanosoma*, and *Spirochetes*. *This fact of a multiple infection in owls together with the normal presence of flagellates in mosquitoes goes to show the great difficulties under which Schau-*

In view of the frequent occurrence of spirochetes in the mouth, stomach, and intestines of higher animals, the presence of these organisms might be expected in the gut of insects. As a matter of fact, several instances of this kind are now known. The three pathogenic spirilla, *Sp. gallinarum*, *Sp. Obermeieri*, and *Sp. Duttoni*, have been found in their insect hosts. The Sergents in 1906, reported the finding of spirochetes in large numbers in a preparation made in 1901, from the digestive tube of a larva of *Anopheles maculipennis*. These measured 8–17 μ (average 13 μ), and showed from 1.5 to 4 weak, spiral turns. At times they were agglutinated. A very similiar, if not identical, organism, *Spirillum glossinae*, was described shortly afterward by Novy and Knapp. It was present in the stomach contents of two tsetse flies (*Glossina palpalis*). The single cell or short form with three to four turns measured 8 μ, while the double cells were about 15 μ long. Another instance of this kind is given by Wenyon,[1] who found spirochetes in lice which infested mice having *Sp. muris*. There was no sign of multiplication, and attempts at infecting mice by means of lice were unsuccessful.

In our own work with mosquitoes we have not been successful in finding real spirochetes, although special search was made for them. In the stained preparations made from several mosquitoes we did find spirochete-like forms but the presence of foreign particles made their nature rather uncertain. We learn through a personal communication from Captain Patton that true spirochetes are very common in the mosquitoes about Madras. It is evident, therefore, that in some localities the genuine spirochetes may be detected in mosquitoes, flies, etc., more readily than in others.

BEHAVIOR OF MAMMALIAN TRYPANOSOMES IN MOSQUITOES.

As bearing upon the question of the multiplication of trypanosomes in the gut of insects, it may be of interest to present some results obtained with *Tr. Lewisi* and *Tr. Brucei*. Wild mosquitoes were allowed to bite infected rats and guinea-pigs, and after a variable

dinn worked while endeavoring to establish the life-cycle of H. Ziemanni. Now that attention is called to the probable existence of a spirochetal disease of owls it is important that a special effort be made to detect the organism in their blood and to demonstrate the action of pure cultures upon clean birds, and their behavior in the gut of mosquitoes.

[1] *Jour. Hyg.*, 1906, 6, p. 583.

length of time the stomach contents were examined and injected into rats and mice. The combined contents of from two to ten mosquitoes were injected into each animal.

No multiplication of these trypanosomes was observed in the stomach even in mosquitoes which were re-fed. The number of trypanosomes ingested rapidly decreased, and dead forms were found after a few hours. In the Lewis mosquitoes, flagellates were found alive as late as 65 and even 114 hours, but these did not resemble either the blood or the cultural forms of *Tr. Lewisi*, and without doubt were ordinary herpetomonads. Unquestionable survivals were noted occasionally as late as 36 and even 48 hours. The *Tr. Brucei* died out more rapidly than the rat trypanosomes, but a few were seen to persist in some mosquitoes as late as 30 and even 36 hours.

Of 16 rats injected with the Lewis mosquitoes, six died from bacterial infection in from three to 11 days, and 10 were under observation for over a month. In none of these rats were trypanosomes detected. The injections were made one and one-half, three, six, nine, and twelve hours after the infective feed.

Of 10 mice inoculated with the Bruce mosquitoes, two developed an infection after an incubation period of eight days. One of these, which received the stomach contents of two mosquitoes, nine hours after feeding, died in 11 days; the other, which received the contents of five mosquitoes 12 hours after feeding, died in 10 days. Two mice which received stomach contents, 30 and 36 hours after feeding, died in four and five days respectively from bacterial infection. Of four rats inoculated with the stomach contents only one became infected after a period of incubation of nine days. This received the stomach contents of three mosquitoes, 14 hours after feeding, and died in 13 days. On looking over our laboratory notes we are unable to find any record of successful infection of mice with mosquitoes which had fed 24 to 36 hours before. Hence the statement to that effect, made in a previous paper, is erroneous. Positive results, it will be seen, were obtained at the end of nine, 12, and 14 hours, but not always. On account of variable conditions in different mosquitoes, the infection experiments do not always succeed in the stated intervals or even in shorter ones. Thus, we obtained failures at the

end of six, 12 and 14 hours, as well as in 18, 24, 30, 36, and 42 hours.

It will be seen from the above that the conditions in the stomach of the mosquito are vastly more unfavorable than in the culture-tube. In our previous studies on *Tr. Brucei*[1] it was shown that the sluggish survivals in the culture-tube (after inoculation with fresh blood) were usually non-virulent after the fourth or fifth day, and that if infection did occur it was only after a long period of incubation. In the above mosquito experiments the survivals failed to infect after 14 hours. The failure to infect with *Tr. Lewisi* is probably due to the same cause. Apropos of these tests, it may be recalled that Bruce was unable to infect dogs by injecting the stomach contents of tsetse flies after an interval of more than half an hour from the time of the infective feed. The fact that the ingested trypanosomes lose their virulence so rapidly in the stomachs of insects indicates a loss of functional activity, especially the power of multiplication and hence such insects cannot play the part of an active host. In view of these unfavorable conditions, it is difficult to see how the mammalian trypanosomes which are no longer able to multiply, even when placed in susceptible animals, could have a life-cycle within such a host.

TRYPANOSOMES OF TICKS.

The detection of trypanosomes by Weber, in the blood of some cattle suffering with Texas fever, and the subsequent finding by Schaudinn of like organisms in restained preparations of the intestinal contents of ticks, taken from infected cattle in Finland, led the latter to set up the working hypothesis that the development of the piroplasmes of cattle and dogs is similar to that of the halteridia. That is to say, Schaudinn was led to believe that the piroplasmes possessed a trypanosomal stage resembling that which he had described for the intracellular parasites of the owl.

In view of the position assumed by him, and its acceptance by some writers, it will be of interest to note that trypanosomes have been recently found at Madras in a dog-tick, *Rhipicephalus sanguineus*, by Captain S. R. Christophers, I. M. S. He found the organisms in only one specimen of the tick, and the slide containing these flagel-

[1] *Jour. Infect. Dis.*, 1904, 1, p. 17.

lates he very kindly placed at our disposal. The preparation, which was made from the lower end of the Malpighian tubule or portion of the rectum, showed a considerable number of well-stained trypanosomes. We propose to designate this flagellate as *Tr. Christophersi*. Another instance of flagellate infection of a tick is mentioned by Doflein, who quotes Leydig as finding them in *Ixodes testudinis*.

From the photographic reproductions given in Plate 13 it will be seen that the flagellates of the dog-tick are typical "cultural" trypanosomes. They are considerably larger than the *Crithidia* and *Herpetomonas* of mosquitoes, and are quite different from the trypanosomes in tsetse flies, as will be seen on comparison with the microphotographs of the latter.[1] They resemble somewhat the cultural forms of certain bird trypanosomes which we have very briefly described under Type 4 in a previous paper,[2] but this of course does not imply that they are derived from avian flagellates. It is more likely that they will be found to come from mammalian forms.

The *Tr. Christophersi* is characterized by a prominent undulating membrane which extends over about half the length of the body. The roundish nucleus lies at about the middle of the cell, and is nearly of the same width as the latter. The small blepharoplast, about 0.7 μ in diameter, is usually close to and on one side of the nucleus, but at times it is found to lie immediately in front. From the widest central portion the body tapers in both directions. Anteriorly it gradually merges with the flagellum, and posteriorly it terminates in a sharp, pointed end very much as in the case of *Tr. Lewisi*. Minute colorless globules are present, especially in the posterior half of the cell. Division is longitudinal and unequal.

The trypanosomes are fairly uniform in size and are all of the same type. The total length is from 30 to 45 μ. The body is usually about 25 μ long, but may vary from 20 to 35 μ. The width is from 2.5 to 4 μ. The length of the free flagellum is 8–12 μ.

The size of these tick flagellates is of itself sufficient to exclude any possibility of their being developmental stages of the *Piroplasma canis*. If there was any doubt on this point, it has definitely been set aside by the recent studies of Captain Christophers, from which

[1] *Jour. Infect. Dis.*, 1906, 3. Plates 15, 16, 17.

[2] *Ibid.*, 1905, 2, p. 292.

it appears that he has succeeded in following out the complete life-history of this organism. His work demonstrates that in the life-cycle of this cytozoon there is no trypanosomal stage. There is therefore very little probability of a flagellate stage being found in the other piroplasmes, cattle, horse, etc. All of the known facts with reference to insect trypanosomes go to show that those present in ticks are either derived from like forms in the blood of animals on which they feed or that they are parasites peculiar to these insects.

RELATION OF THE INSECT FLAGELLATES TO THE BLOOD TRYPANOSOMES.

This subject has been discussed (p. 234), in part, in connection with the account of the various investigations on insect parasites. It has been shown that abundant caution must be used in the interpretation of such findings. A natural parasitism of the intestinal tract is clearly a widespread and common condition, and it must be conceded that up to the present time no incontestable evidence has been produced to show that the sanguicolous trypanosomes are able to grow and multiply in insects. The facts, such as they are, indicate that the biting insects merely transmit the surviving unaltered trypanosomes which they have taken up with the blood, and that such transmission can occur only during the few hours following the infective feed. The innocuousness of biting insects, such as *Glossina morsitans*, *Gl. palpalis*, rat lice, etc., after they have digested the infected blood, can be interpreted in only one way, namely, that the ingested trypanosomes have died out or are about to do so.

The conditions in the gut of insects are comparable with, but hardly as favorable as, those which are offered in the culture-tube. Knowing the difficulties encountered in the cultivation of *Tr. Brucei*, *Tr. gambiense*, etc., it will be readily understood that even greater ones must exist in the intestinal tract. Hence it is that surviving forms of these organisms are rarely found in the gut after 24 to 36 hours following their introduction with the blood. The more rapid the digestion and absorption of the food, the more rapidly will such organisms perish in the digestive tube.

In the case of *Tr. Lewisi*, *Tr. avium*, etc., which may be looked upon as nearly "saprophytic" on account of the extreme ease with

which they can be grown in the test-tube, it is evident that they are more likely to take on the "cultural" form, and thus adapt themselves to the new conditions than are the truly pathogenic trypanosomes. For this reason it is conceivable that these trypanosomes may multiply in the gut of lice, mosquitoes, etc., but the proof is as yet wanting.

The only instance which we have of the multiplication of the blood trypanosomes in the gut of sanguivora is that afforded by the leeches. The large quantity of blood taken up, the anti-coagulative action of the secretions, the slow digestion, and the low temperature are factors which enable trypanosomes of the type mentioned above to multiply in this intestinal tract, and thus, so to speak, secure a foothold. Hence a leech, once infected, is capable of transmitting the infection to eels, fish, and frogs, etc., even after some weeks have elapsed from the time of the infective feed.

It has been repeatedly shown that the flagellates present in the intestines of insects and leeches present the characteristics of cultural forms of trypanosomes, and this fact is now definitely proven for *Crithidia* and *Herpetomonas*. These organisms do grow and thrive in the test-tube vastly better than in the mosquito, and the rich cultures thus obtained afford the best material for the study of "the parasite's life-history and biology." The *ex cathedra* condemnation by Woodcock of the cultivation method on the ground "that this is not a zoological method of research" will hardly be accepted by the true investigator who is not bound down by set forms and procedures. Scientific progress demands the application of new methods, and though "the trypanosomes are not bacteria," it is safe to say that the cultivation method as applied to these organisms will yield results which might be utilized to advantage by the morphologist.

Bearing in mind the fact that the blood trypanosomes when grown *in vitro* lose their original form and present cultural characteristics which ally them to the gut flagellates, it will be seen that the blood form in all probability represents the latest adaptation, to a new environment, of organisms which originally are derived from the intestine of insects. The adaptation to living matter is obviously the sequel of a previous existence on dead matter, and for that reason the intestinal or cultural forms instead of being involutions or degenera-

tions, closely represent, as Brumpt first pointed out, the ancestral or primitive type of the sanguicolous trypanosomes.

RELATION OF INSECT FLAGELLATES TO CYTOZOA.

A brief statement of this question, by way of a summary, will not be out of place in connection with the foregoing topic. The demonstration of at least two distinct flagellates, *Crithidia* and *Herpetomonas*, in all stages of ordinary mosquitoes, goes to show beyond any doubt that these forms bear no immediate phylogenetic relation to the intracellular parasites. The recognition of this fact more than ever invalidates the observations of Schaudinn on halteridium and leucocytozoon. It is imperative that his work should be repeated with the same species of bird (*Athene noctua*), the same species of mosquito (*Culex pipiens*), and if possible in the same locality, namely Rovigno, and that under experimental conditions which will leave no room for criticism. The several possible sources of error have already been pointed out (p. 230), and until that work is confirmed it is unwise to give it undue prominence and to base on it far-reaching conclusions as some zoologists have done.

We are firmly of the belief that the large typical trypanosome which is commonly present in owls corresponds closely to the large form or "female" of *Tr. avium* which we have described and illustrated in a previous paper.[1] The similarity will be evident on comparing our photographs with the one given by Nocht[2] and designated as the female form of *Spirochaeta Ziemanni*. Hence our further belief that the owl trypanosome is no more related to the *H. Ziemanni* than is *Tr. paddae* to the halteridium which often accompanies it. Our work on the "Bird Trypanosomes" has shown conclusively that the blood trypanosomes are definite species and are not stages in the life-history of cytozoa. In our next paper we expect to show beyond doubt that *H. Ziemanni*, in the blood, has a different development from that worked out by Schaudinn. Moreover, we have allowed "raised" mosquitoes to feed on hawks infected with *H. Ziemanni*, and have not been able to secure the "spirochetes" or other evidence of flagellate stages. It may be added, parenthetically,

[1] *Jour. Infect. Dis.*, 1905, 2, Plate 3.

[2] Kolle and Wassermann's *Handbuch*, Ergänzungsband, Erstes Heft, 1906, Plate 3, Fig. 3.

that we have not been able to detect flagellates in a large number of larvae which were examined for that purpose, and hence this may account for the negative results just mentioned. Had we allowed "wild" mosquitoes to feed on the infected hawks, we should have obtained, without doubt, a flagellate infection as did Schaudinn and the Sergents. The fact that the latter used "raised" mosquitoes does not exclude the presence of intestinal flagellates, since it has been shown that in the warm countries infection of larvae is a not uncommon occurrence. The present study shows that the ordinary "wild" mosquitoes do harbor herpetomonad forms which conform fully to the "spirochetes" which the Sergents considered identical with those described by Schaudinn (p. 253). The relation of Töpfer's owl spirochete to *H. Ziemanni* has been discussed on p. 259.

The position of *Trypanosoma noctuae* is essentially like that of *Spirochaeta Ziemanni*. Inasmuch as the halteridium infection of owls and hawks in Michigan is much less frequent than that with the leucocytozoon, we have not been able to make any feeding experiments with mosquitoes in this type of infection. The study of the natural mosquito parasites, however, shows the same possible source of error and leads to the conclusion that the so-called *Tr. noctuae*, instead of being a developmental form of the intracellular halteridium, is a common inhabitant of the gut of mosquitoes, and is probably a mixture of crithidian and herpetomonad forms.

It is certainly significant that, in spite of the stimulus which Schaudinn's observations have given to the examination for flagellate stages, no one has yet demonstrated such stages for any one of the intracellular parasites. Thus, Billet's supposition that the *Tr. inopinatum*, in frogs, was the flagellate stage of a *Drepanidium* has been effectually disproved by Brumpt. The guarded supposition of Koch that the trypanosome present in crocodiles is in relation either with the accompanying hemogregarine or with the tsetse-fly flagellates (*Tr. Grayi*) will hardly stand the test of actual experiment. Lastly, the work of Thiroux has clearly shown the absence of any genetic relation between *Halteridium* and *Tr. paddae*, and the recent studies of Christophers are equally conclusive as to the absence of a flagellate stage in the life-cycle of *Piroplasma canis*. The "flagellate-like"

forms recently described by Kinoshita for this organism have very little that is suggestive of trypanosomes.

ISOLATION OF TRYPANOSOMES FROM MIXED CULTURES.

F. G. Novy and R. E. Knapp.

In general, it may be said that bacteria once introduced into a culture of trypanosomes tend to outgrow and check the development of the flagellates. It is probable that this injurious action is due largely to the production of poisonous products or to an alteration in the reaction of the medium. The antagonism between certain bacteria and these flagellates is deserving of attention. In exceptional instances, however, the bacteria exert little or no interference, and may even be apparently beneficial. While in the former instance the trypanosomes soon die out, in the latter the mixed culture can be kept, by frequent transplantation, almost indefinitely. We have, for example, maintained a mixed culture of *Tr. Mesnili* associated with a coccus for nearly a year. Similarly, *Crithidia fasciculata*, which was isolated from a mosquito along with a yeast, was cultivated in this mixed form for nearly six months. Likewise, the *Tr. culicis* was grown in mixed culture with a minute diplo-bacillus for nearly five months. In these instances the foreign organisms did not alter, or but very slowly, the color of the blood agar, and it would seem from other trials as if a rapid destruction of hemoglobin by the accompanying bacteria was an injurious factor.

The isolation of trypanosomes from the gut of insects in a pure condition is hardly to be expected, owing to presence of various bacteria and yeasts. There is a possibility, however, of obtaining a "pure, mixed" culture—that is, where the flagellate is associated with only one kind of foreign organism—and such cultures as seen from the above examples are more favorable for transplantation than when a number of different species are present. The need of strictly pure cultures is soon recognized when it is desired to study the pathogenic action of the trypanosomes.

We have attempted to secure a separation by three different methods. In the first method fairly good results were obtained by inoculating the culture medium some 2 or 3 cm. above the condensation fluid, and then keeping the tubes in an almost horizontal position.

This was done with the object of allowing the motile trypanosomes to travel away from the accompanying bacteria or yeasts, and thus reach the fluid at the bottom of the tube. Several times almost pure cultures were obtained in this way, especially in the case of the *Crithidia* which was associated with a large yeast. Fairly good results were also obtained with the *Herpetomonas*, but in that instance the minute bacillus was more readily carried by the sticky flagellates into the condensation fluid. In the presence of motile or slimy bacteria this method fails entirely.

In the second method the attempt was made to utilize the marked aerotropism of the trypanosomes. As has been pointed out, the cultural flagellates tend to gather in compact layers about minute bubbles of air. On removing such air-globules by means of a capillary it would seem as if almost pure transplants could be obtained. The method, however, was not tested sufficiently to show its practicability.

The method which gave perfectly satisfactory results was as follows: By means of a glass spatula, made by drawing out the end of a glass rod, a little of the mixed culture was spread in a series of streaks over six Petri dishes containing solidified blood agar. Ordinary agar may be used in the first three dishes, since the desired dilution is not attained until in the last three. The form of Petri plate known as the "Kriegsministeriums-Modell," made by Greiner and Friedrichs, is particularly adapted for this purpose, inasmuch as it can be effectively sealed by means of a wide rubber band. The sealed dishes were then set aside at room temperature for 10 or 12 days. The last plate or two of the series was found to contain isolated colonies of trypanosomes, which could then be transplanted in the usual usual way to the test-tube. By this means we were able to secure a pure culture of *Crithidia*, and were almost successful with *Herpetomonas*. Without doubt this method will be found useful in future studies of the flagellates found in the gut of insects and other sanguivora.

SUMMARY.

The results and conclusions of this study can be recapitulated as follows:

1. Of the "wild" mosquitoes examined by us approximately 15 per cent in one year, and about 5 per cent in that following, were

found to be infected with flagellates belonging to two easily differentiated types, *Crithidia* and *Herpetomonas*. The percentage of infections is influenced largely by local and seasonal conditions.

2. By the cultivation method it has been possible to isolate two of these flagellates, namely *Crithidia fasciculata* and *Tr. culicis;* and it has been shown that the plate method is applicable for the separation of trypanosomes from the accompanying bacteria and yeasts.

3. The organisms obtained *in vitro* correspond to those observed *in vivo*, and hence the intestinal types represent true cultural forms.

4. The two types are common in other insects, and, instead of being classed as distinct genera, they should be placed under the trypanosomes.

5. The mosquito flagellates are not stages of intracellular organisms, but are probably parasites peculiar to the insects.

6. The inoculation of available experimental animals with the *Crithidia* and *Herpetomonas* has given negative results.

7. The *Tr. noctuae*, as described by Schaudinn, presents the mixed characteristics of *Crithidia* and *Herpetomonas*, while that of the Sergents agrees with the *Herpetomonas;* as such, it does not represent a stage of the *Halteridium*.

8. The cultivation by Töpfer of a spirochete from the blood of an owl, presumably identical with Schaudinn's *Sp. Ziemanni*, indicates the existence of a new spirillosis. The forms which the Sergents identified with *Sp. Ziemanni* were probably not spirochetes but trypanosomes, more especially the long form of a *Herpetomonas*. The conclusion seems justified that neither the flagellates and spirochetes found in mosquitoes nor the trypanosomes and spirochetes found in the blood of owls are related to the leucocytozoon.

9. The trypanosomes which are at times present in ticks are not developmental forms of *Piroplasma*. It has already been shown that the trypanosomes in tsetse flies are not related to *Tr. gambiense* or *Tr. Brucei*.

10. The possibility of the trypanosomes of birds and other vertebrates developing in the gut of insects, while not excluded, has not been demonstrated. In the mosquito, *Tr. Lewisi* and *Tr. Brucei* lose their infectiousness, more or less rapidly, but the enfeebled organism may survive in the gut for 36–48 hours. The conditions

in the digestive tube of insects is not as favorable as in the test-tube, and this fact goes to show that insect carriers, such as tsetses, are mere passive hosts.

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EXPLANATION OF PLATES.

The accompanying photographs are all taken at the uniform magnification of 1,500 diameters, and, as such, they are directly comparable with those of other cultural trypanosomes previously given. The preparations were stained by a modified Romanowsky method.

PLATE 7. CRITHIDIA FASCICULATA.

(As found in mosquitoes.)

Fig. 1.—*Crithidia* from mosquito No. 99.

Fig. 2.—Same preparation, showing *Crithidia* and *Herpetomonas*. Note the square anterior end and the short flagellum of the former; the diplosome posterior to the nucleus in the latter.

Fig. 3.—Elongated *Crithidia* from the same preparation.

Fig. 4.—*Crithidia* from mosquito No. 56.

Fig. 5.—*Crithidia* from mosquito No. 46.

Fig. 6.—*Crithidia* from a culture nine days old (Gen. 1); compare Figs. 5 and 6; the former is derived from a mosquito, the latter from a culture. Note the similarity in size and structure, showing that the wild *Crithidia* are really cultural forms *in vivo*. Compare also with cultural forms shown on Plates 2 and 3.

PLATE 8. CRITHIDIA FASCICULATA.

(Cultural forms of Strain No. 10.)

Figs. 1 and 2.—Multiplication rosettes, showing large and small cells. Note the unstained channel in some showing position of whips which are directed centrally; also the small round or oval blepharoplast adjoining the nucleus. Nine-day culture, Gen. 1.

Fig. 3.—Dividing form, final stage; showing two nuclei, two blepharoplasts, and two flagella, one of which is longer than that of the parent cell. Three-day culture, Gen. 39.

Fig. 4.—Part of a rosette of elongated *Crithidia* with flagella directed centrally; note the stippling, Gen. 32.

Fig. 5.—Group of short *Crithidia* from the same preparation as Figs. 1 and 2. Note the truncated anterior end.

Fig. 6.—Elongated *Crithidia* from the same preparation as preceding.

PLATE 9. CRITHIDIA FASCICULATA.

(Cultural forms from Strain No. 10. Figs 1, 2, and 3 are from a preparation of the 20th generation or sub-culture.)

Fig. 1.—An agglutination group of *Crithidia*.

Fig. 2.—Short oval form showing square anterior end, blepharoplast adjoining the nucleus, and a short flagellum, bent within the cell.

FIG. 3.—Short, thick form with concave anterior end; blepharoplast and flagellum as before.

FIG. 4.—Goblet-shaped *Crithidia*. Note the square anterior end and the constriction posterior to the nucleus. Also the dark granules at posterior end. Compare with the constricted forms shown in Schaudinn's. Figs. 1*b*, 2*b*, and 4*b*. From a third generation, two days old.

FIG. 5.—Normal and goblet-shaped *Crithidia* from the same preparation as preceding.

PLATE 10. TRYPANOSOMA (Herpetomonas) CULICIS.

(As found in mosquitoes; compare with cultural forms on Plate 12.)

FIG. 1.—Pale slender form of medium length; showing flagellum, blepharoplast, nucleus, and posterior diplosome or acrosome. From mosquito No. 52. Compare with cultural form shown in Fig. 1, Plate 12.

FIG. 2.—Longer form from same preparation; showing undulating membrane, flagellum, blepharoplast, nucleus, and posterior body. Compare with cultural form shown in Fig. 2, Plate 12.

FIG. 3.—From same preparation as preceding. Note the short new flagellum (?) indicating initial division.

FIG. 4.—A medium form, showing two diplosomes or pairs of posterior bodies. From mosquito No. 99.

FIG. 5.—A similar cell from mosquito No. 52, showing two diplosomes, and possibly a third against the nucleus.

FIG. 6.—Small form (male ?) from same preparation as preceding.

PLATE 11. TRYPANOSOMA (Herpetomonas) CULICIS.

(As found in mosquitoes and in cultures.)

FIG. 1.—Rounded-up form from mosquito No. 4. Note the two large diplosomes between the blepharoplast and nucleus.

FIG. 2.—Short divisional form, showing two flagella, two blepharoplasts, two nuclei, and two diplosomes or pairs of posterior bodies. From mosquito No. 52.

FIG. 3.—Cultural form from Strain No. 29, Generation 17, showing early stage of division. Note the thickening of the base of flagellum which is due to the presence of a short new whip; division of the blepharoplast; undivided nucleus and the presence of two diplosomes or pairs of posterior bodies.

FIG. 4.—Divisional form from same preparation as preceding; showing two flagella and two nuclei with rather indistinct posterior bodies. Two blepharoplasts are present, but one lies partly over the other.

FIG. 5.—Divisional form from same preparation, showing two prominent blepharoplasts as well as two flagella and two nuclei.

FIG. 6.—Divisional form from same preparation, showing complete division; prominent posterior bodies and blepharoplasts.

PLATE 12. TRYPANOSOMA (Herpetomonas) CULICIS.

(Cultural forms from the 17th generation of Strain No. 29.)

FIG. 1.—Pale, slender form, corresponding to that shown in Fig. 1, Plate 10. Note the prominent diplosome.

FIG. 2.—Longer form, showing undulating membrane and posterior diplosome. Compare with the mosquito form shown in Fig. 2, Plate 10.

Fig. 3.—Rounded-up form, showing flagellum, blepharoplast, nucleus, and prominent diplosome. Compare with Fig. 1, Plate 11.

Fig. 4.—Very short and long male and indifferent forms; also a rounded-up cell as in preceding figure, but showing two diplosomes or pairs of posterior bodies.

Fig. 5.—Large and rather rare form (female ?) with two nuclei. The diplosomes are in the anterior portion.

Fig. 6.—Large (female ?) form, similar to preceding, in process of division.

PLATE 13. TRYPANOSOMA CHRISTOPHERSI

(From the dog-tick, *Rhipicephalus sanguineus*, in preparation of Captain S. R. Christophers, I. M. S.)

Figs. 1, 2, and 3.—Forms showing prominent undulating membrane and blepharoplast anterior or lateral to the nucleus. Note the sharp posterior end and the presence of globules.

Fig. 4.—The same, with less prominent undulating membrane.

Fig. 5.—Form showing rounding-up of posterior end, also large globules.

Fig. 6.—Unequal longitudinal division.

PLATE 7.

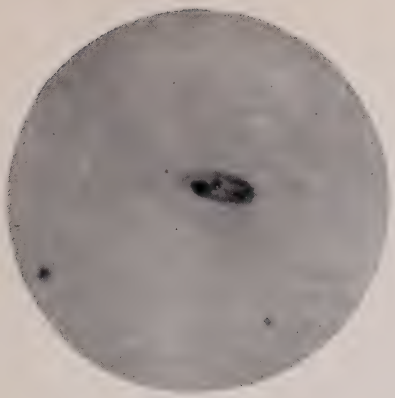

FIG. 1.

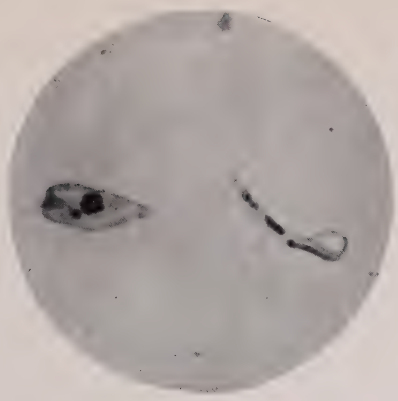

FIG. 2.

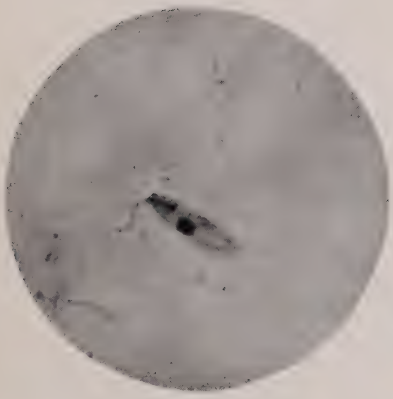

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

PLATE 8.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

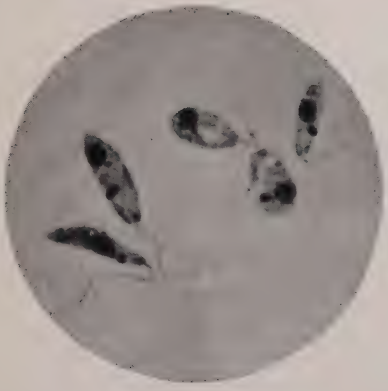

FIG. 5.

FIG. 6.

PLATE 9.

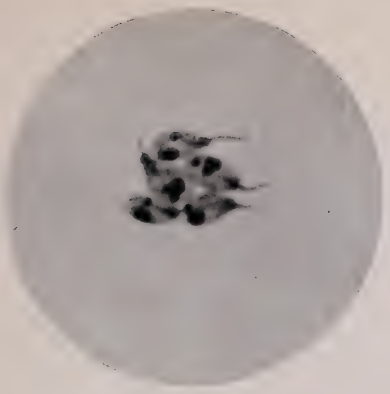

FIG. 1.

FIG. 2.

FIG. 5.

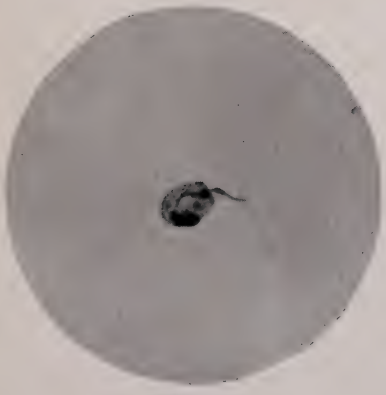

FIG. 3.

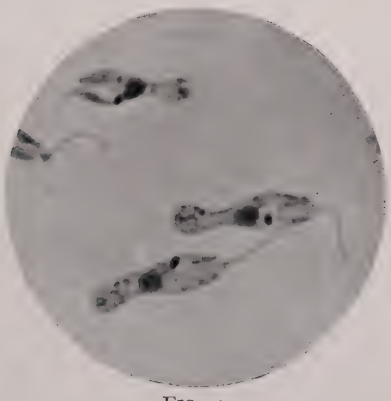

FIG. 4.

PLATE 10.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

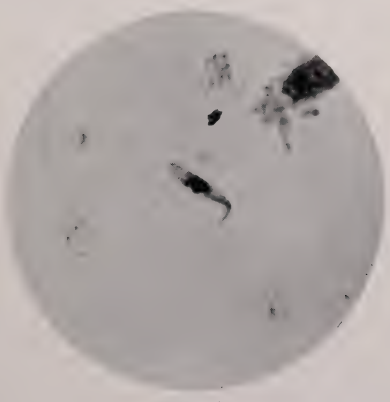

FIG. 6.

PLATE II.

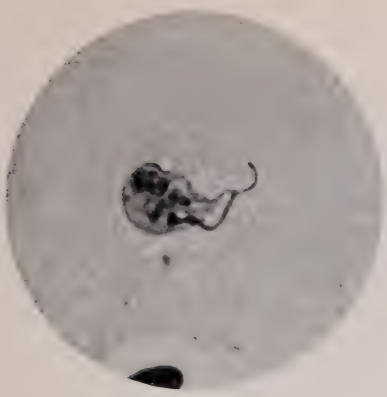

FIG. 1.

FIG. 2.

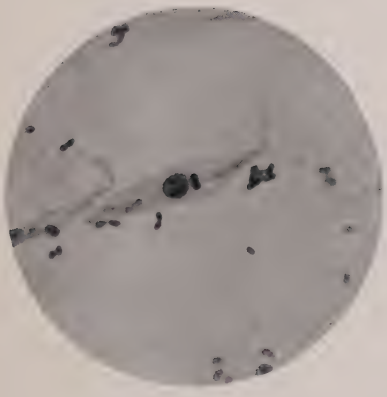

FIG. 3.

FIG. 4.

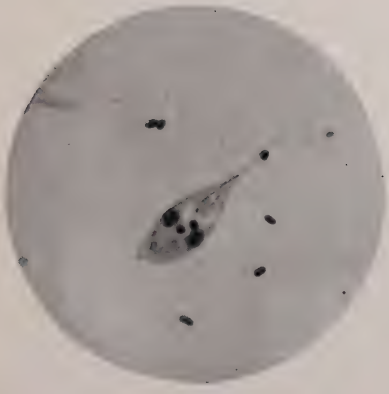

FIG. 5.

FIG. 6.

PLATE 12.

FIG. 1.

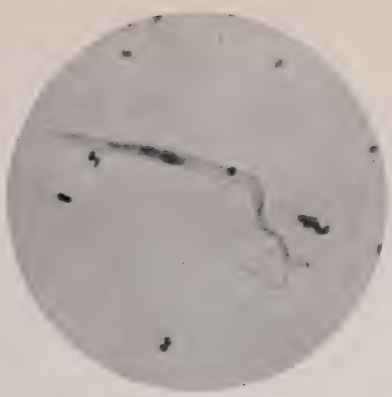

FIG. 2.

FIG. 3.

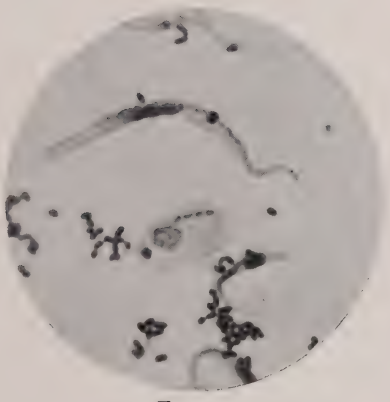

FIG. 4.

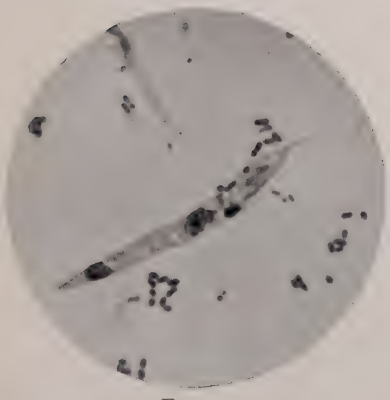

FIG. 5.

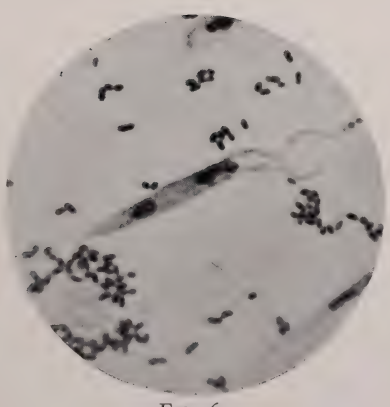

FIG. 6.

PLATE 13.

FIG. 1.

FIG. 2.

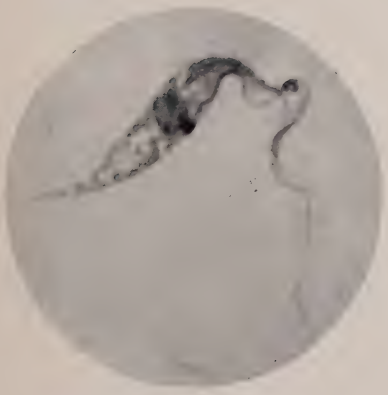

FIG. 3.

FIG. 4.

FIG. 5.

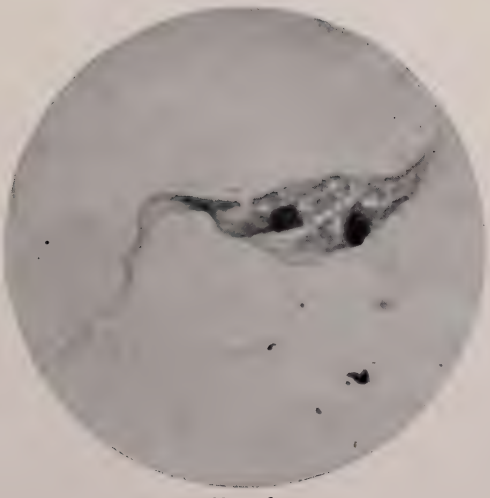

FIG. 6.

A STUDY OF THE NATURE OF STREPTOLYSIN.*

SECOND CONTRIBUTION.

GUSTAV F. RUEDIGER.

(From the Memorial Institute for Infectious Diseases, Chicago.)

IN 1901 Besredka[1] succeeded in getting filtrates of streptococcus cultures which were strongly hemolytic for red corpuscles of many animals. To get this hemolysin in solution he grew a highly virulent streptococcus in heated rabbit serum for 20 to 24 hours and passed the culture through a Chamberlain filter. He made a study of this substance and found that it does not pass through a dialysing membrane, that it deteriorates with age, and that it is destroyed by heating at 70° for two hours.

In the previous publication I[2] have verified and extended Besredka's observations. In that paper it was shown that streptolysin is destroyed by peptic digestion, that it is neutralized by a weak solution of formaldehyde, and that it behaves like a substance having a haptophore and a toxophore group firmly linked together. That is, at a temperature of 5° to 6° C. it attaches itself to the red corpuscles so firmly that it cannot be washed away by repeated washings of the corpuscles in cold salt solution. No hemolysis takes place as long as the temperature is kept below 6° C., but if the temperature of the suspension of washed treated corpuscles is raised to 30° to 40° C., the latter are rapidly hemolysed. It was shown further that heated chicken serum is quite strongly antihemolytic when added to streptolysin before the addition of the red corpuscles. If, however, the streptolysin is bound to the corpuscles in the cold, and the corpuscles are then washed and suspended in cold salt solution, the addition of chicken serum to this suspension does not prevent hemolysis when the temperature is brought up to 30° to 40° C. But if, instead of chicken serum, a trace of zinc chloride or zinc sulphate is added to such a suspension of washed treated corpuscles, no hemolysis takes place when the temperature is raised. That is, the chicken serum seems

* Received for publication September 5, 1906.

[1] *Ann. de l'Inst. Pasteur*, 1901, 15, p. 880.

[2] *Jour. Am. Med. Assoc.*, 1903, 41, p. 962.

to affect only the haptophore group, while the zinc salt ($ZnCl_2$) destroys or neutralizes the toxophore group after the streptolysin has been bound to the red corpuscles.

During the last three years a number of facts have been ascertained which throw additional light upon the nature of streptolysin.

Serum culture in an amount of 10 c.c. was evaporated to dryness by passing a current of dried air over it at room temperature. The residue was ground up in a mortar, further dried in a dessicator for 24 hours, and sterilized in dry heat at 150° C. for 30 minutes. The dried powder only partially dissolved in 0.85 per cent NaCl solution, but the clear centrifugated solution hemolysed rabbit corpuscles in two to three hours.

Attempts were made to get streptolysin in pure form—that is, to free it from the proteids of the serum. These attempts have not been wholly successful, but the results are interesting and worth recording. Twenty c.c. of filtered serum culture which was strongly hemolytic were saturated with magnesium sulphate at 35° C. and allowed to stand at this temperature for two hours. The precipitate was now filtered out, washed on the filter with a saturated solution of magnesium sulphate, and redissolved in 20 c.c. of 0.75 per cent NaCl solution. This solution and the filtrate were dialysed in separate dialysers for 24 hours against a continuous stream of distilled water. After dialysis enough NaCl was added to each lot to make a 0.75 per cent solution,[1] and each portion was tested for streptolysin with rabbit corpuscles. The test showed that the dissolved precipitate is strongly hemolytic, while the filtrate has no hemolytic powers; that is, the streptolysin is precipitated quantitatively with the globulin by saturation with $MgSO_4$ at 35° C. This result has also been obtained when the globulin was precipitated by mixing equal volumes of streptolysin (filtered serum culture) and a saturated solution of ammonium sulphate (saturated at 35° C.) and filtering after one to two hours. If the mixtures are filtered immediately after adding the salt, the filtrate contains some hemolysin, although the greater part of it is in the precipitate.

I now tried fractional salting-out of the globulin and tested each

[1] A part of the globulin usually is precipitated and may not be completely redissolved in the salt solution.

fraction for streptolysin. Euglobulin was precipitated by adding 5 c.c. of a saturated solution of $(NH_4)_2SO_4$ to 10 c.c. of a strongly hemolytic filtrate of a serum culture of streptococcus. After standing two hours the precipitate was collected on a filter and washed with a one-third saturated solution of $(NH_4)_2SO_4$ and dissolved in 0.75 per cent NaCl solution. The filtrate was now treated with 5 c.c. more of saturated $(NH_4)_2SO_4$ solution and the second precipitate (pseudoglobulin) collected on a filter, washed with a half-saturated solution of $(NH_4)_2SO_4$, and dissolved in 0.75 per cent NaCl solution. The solutions of euglobulin and of pseudoglobulin were each dialysed for 24 hours and (after adding enough NaCl to make a 0.75 per cent solution) tested for streptolysin with rabbit corpuscles. Both solutions were found to be quite strongly hemolytic.

Ten c.c. of streptolysin were now diluted with 90 c.c. of distilled water and a stream of CO_2 passed into the solution for one hour. The precipitate of insoluble globulin was centrifugated out of the liquid, washed twice with distilled water which had been saturated with CO_2, and then dissolved in 0.75 per cent NaCl solution. This solution was hemolytic for rabbit corpuscles, as was also the diluted streptolysin from which the insoluble globulin had been separated.

Very similar results were obtained by precipitation of the insoluble globulin by dialysis. Five c.c. of streptolysin were mixed with an equal volume of saturated solution of $(NH_4)_2SO_4$, and after standing two hours the precipitate was collected on a filter, washed with a half-saturated solution of $(NH_4)_2SO_4$, and dissolved in 0.75 per cent NaCl solution. This solution was dialysed against distilled water for 48 hours, at which time it contained a heavy precipitate. The precipitate was collected on a filter, washed three times with distilled water, and dissolved in 0.75 per cent NaCl solution. Not all of the precipitate went into solution, but the solution was found to be hemolytic for rabbit corpuscles. The filtrate containing the soluble globulin also had marked hemolytic powers.

Several attempts were made to extract the streptolysin from the filtered serum cultures by treatment with ether, alcohol, and chloroform, but all of these experiments gave negative results.

The normal serum of some animals, as for instance the chicken the goat, and the horse, has a decided antistreptolytic effect. This

antistreptolytic action may be represented by a curve if falling quantities of the serum are added to tubes containing a definite quantity of streptolysin and washed corpuscles, and the percentages of hemolysis are taken as ordinates and the amounts of serum as abscissas. Fig. 1 shows such a curve obtained with heated goat serum as the antistreptolysin. Each tube contained 0.4 c.c. streptolysin, 0.2 c.c. of a 20 per cent suspension of washed rabbit corpuscles, falling quantities of heated goat serum, and enough 0.85 per cent NaCl solution to make 2 c.c. The tubes were incubated at 36° C. for two hours, during which time they were shaken once. At the end of the incubation they were centrifugated and the percentages of hemolysis determined with the aid of a calorimetric scale. If the percentage of hemolysis is carefully determined, the resultant curve is a fairly regular one.

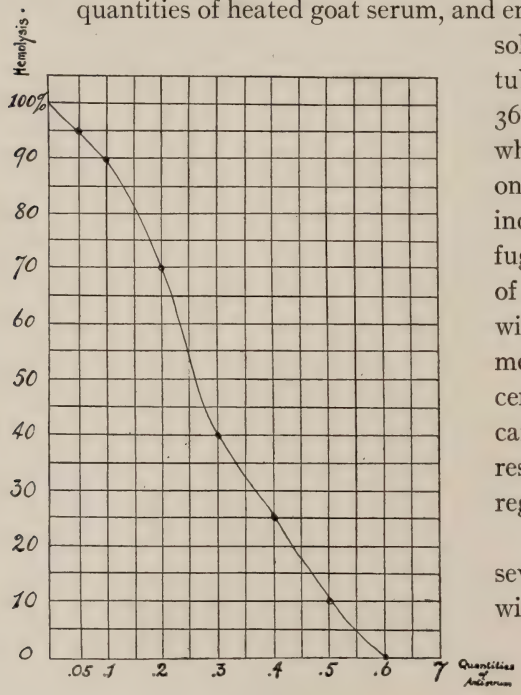

FIG. 1

A goat, a chicken, and several rabbits were injected with gradually increased quantities of streptolysin, but no specific antistreptolysin could be produced. The sera of several rabbits and of a horse which had been immunized against a virulent streptococcus were tested for antistreptolysin, but no definite increase in antistreptolytic power over that of normal sera could be detected. The sera of two rabbits seemed to have a slightly greater antistreptolytic action than the normal control, but the difference was so small that it may well have been due to a variation in the antistreptolytic power of the normal serum.

Breton[1] and very recently Tchitchkine[2] claim to have been able to produce a specific antistreptolysin by immunizing rabbits with a virulent streptococcus. The antistreptolytic power of their sera was very little greater than that of the normal serum, and it seems likely, therefore, that they were not dealing with a specific antibody, but with the inhibitive action of normal serum. This view has already been expressed by Besredka[3] in regard to the antistreptolysin described by Breton.

SUMMARY.

In summarizing we can say that streptolysin is a complex organic substance which has a haptophore and a toxophore group.

It is intimately associated with the globulins of the serum in which it is produced and has not yet been separated from them. It can be separated quantitatively from the serum albumin by saturation with magnesium sulphate at 35° C. or by half-saturation with ammonium sulphate, and it seems not unlikely that it is a globulin.

It is neutralized by a weak solution of formaldehyde and is destroyed by peptic digestion.

It cannot be extracted from the serum with alcohol, ether, or chloroform.

It does not pass through a dialysing membrane, but is destroyed by heating at 70° C. for two hours (Besredka).

Chicken serum, goat serum, and horse serum have marked antistreptolytic properties.

Attempts to produce a specific antistreptolysin have not been successful.

[1] *Compt. rend. de la Soc. de biol.*, 1903, 55, p. 886.

[2] *Ann. de l'Inst. Pasteur*, 1906, 20, p. 506.

[3] *Ibid.*, 1903, 1, p. 575.

A SUBSTITUTE FOR POTATO AS A CULTURE MEDIUM.*

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THE appearance of bacterial cultures on potato, which in the early days of bacteriology was considered of diagnostic value, has been shown to be subject to considerable variation. The cause of this variability is sometimes due to differences in composition and reaction of potatoes of various origin. The "invisible" film of *B. typhosus* is an example of the unreliability of potato as a substratum. On potatoes of slightly acid reaction there is a hardly visible growth; if the reaction is alkaline, however, there is abundant growth, which resembles that of other bacteria. The "honey" appearance of *B. mallei* also remains indistinct, if the potato is more acid than usual. Still, most bacteriologists adhere tenaciously to the use of potato as a culture medium, because certain characters develop better on this medium than on any other. Pigments especially often develop abundantly on potato, and frequently appear on this medium only. The secretion of an amylolytic enzym can be demonstrated only on a starch-containing medium. A substratum which enables microorganisms to exhibit these faculties to advantage must, therefore, be of some value.

Media have been prepared by expressing the juice of potato and then adding other ingredients. Although this method permits the adjustment of the reaction, there is nothing gained in knowledge of the chemical composition. I have been able to find only one attempt to substitute a synthetic medium for potato, the "starch jelly" of E. F. Smith.[1] This is prepared by filling 2 grams of starch, freed from impurities by repeated washing in water, into dry culture tubes and adding 10 c.c. of a solution of ammonium sulphate, sodium asparaginate, magnesium sulphate, sodium chloride, dipotassium hydrogen phosphate, calcium chloride, sodium sulphate, or in place of this solution, Uschinsky's medium, omitting the glycerin. The tubes are then heated in a Koch inspissator in a slanting position for two or three

* Received for publication February 1, 1907.

[1] *Proc. Amer. Assoc. Adv. of Sci.*, 1898, 47, p. 411.

hours on each of five or six consecutive days at a temperature of 75° to 85° C. The starch swells and forms a jelly. By this long exposure to heat the water evaporates and has to be replaced. The resulting medium breaks easily and has a rough surface.

This paper came to my attention after I had experimented for several months with a medium, the preparation of which is simpler than the one described above. Starting with a composition suggested by the results of chemical analyses of potato, it was found by a series of cultures that some constituents were of no value, and that the quantity of the necessary constituents might be reduced. The following method of preparation has been found the most advantageous and permits considerable latitude for varying the composition. Fifteen grams of agar are dissolved by heat in about 600 c.c. water. A solution of the following salts in 200 c.c. water is then prepared:

Asparagin	5 gr.
Dipotassium hydrogen phosphate	2
Disodium hydrogen phosphate	2
Magnesium sulphate	2
Calcium chloride	2
Ammonium lactate	2

This solution, in which a fine precipitate is formed, is added to the hot agar solution, 10 grams of peptone are added, and the whole mixture filtered after the reaction, which is about 5 per cent acid, is brought to the neutral point with phenolphthalein as an indicator. To the hot filtered solution a suspension of 30 grams of washed starch, made perfectly homogeneous in a mortar, is gradually added with constant stirring. The mixture is then brought to near the boiling-point and finally weighed. The total should weigh 1,000 grams. The medium is tubed and sterilized in the autoclav for five minutes at 120°, and is cooled in a slanting position. The salts used in the medium are the principal ones contained in potato according to chemical analysis.

This mode of procedure may be varied in several ways. By the mode of preparation described a jelly is obtained which is slightly opaque, homogeneous, smooth, and of sufficient firmness to retain the slant desired. If the salt solution is added after filtration of the agar, the white precipitate will be held in suspension by the swelling starch, and, if the starch has not been heated too long, a whitish, opaque medium results, which shows pigments to great advantage.

The addition of glucose increases its nutritive value, but brings out no amylolytic activity of the microorganisms.

I claim the following advantages for this medium over ordinary potato:

1. The composition is always the same, and the reaction may be adjusted to suit the purpose of the study.

2. The chief nutrient substances of potato, especially starch, are in a highly assimilable form.

3. The disadvantage of potato—i. e., variability in composition—is avoided. The advantages—i. e., pigment formation, amylolytic action, and gas formation—are not only preserved, but are more pronounced than on potato.

4. Diffusible pigments are readily differentiated from non-diffusible ones.

5. The preparation is simple, and the cumbersome potato tube is rendered superfluous.

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HUMAN PNEUMOCOCCAL OPSONIN AND THE ANTI-OPSONIC SUBSTANCE IN VIRULENT PNEUMOCOCCI.*†

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INTRODUCTION.

IN a previous article[1] I have shown that the pneumococcidal action of pneumonic and other blood is the combined result of opsonification, phagocytosis, and intraphagocytic destruction. The observations were made upon avirulent pneumococci *in vitro*, and hence under conditions widely different from those in a pneumococcus infection in which it concerns virulent pneumococci which resist opsonification by human serum. No less than 75 strains of pneumococci from the blood in pneumonia have been shown to be insusceptible to phagocytosis when first isolated, no matter whether the serum used was obtained from normal persons or from patients in various stages of pneumonia. This resistance to phagocytosis is associated with virulence for rabbits and guinea-pigs. In order to define better the actual

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† This work was carried on with the aid of the Dane-Billings' Fellowship in Medicine of Rush Medical College, Chicago.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 683.

rôle of phagocytosis in pneumococcus infection it therefore seemed important to determine the exact relation between opsonins and virulent pneumococci.

FILTERABILITY OF OPSONINS.

In order to study the exact relation between pneumococci, virulent and avirulent, and the opsonin in human serum it was found necessary to develop a reliable technique for experiments on the absorption of opsonin by various bacteria. To avoid error in absorption experiments the bacteria must be separated completely from the serum. For this purpose the centrifuge has been used,[2] but prolonged centrifugalization is time-consuming, and unless great speed is obtainable it is often also inefficient. It was therefore determined to test the filterability of opsonin, and it was found that opsonin is not appreciably diminished by passing serum through porcelain filters. At first a so-called Maasen filter with very small pores was used, so that with a pressure of 25 pounds several hours were required to obtain a small quantity of serum. Subsequently coarser filters have been used requiring from 10 to 30 minutes to deliver 0.5 to 2 c.c. or more of serum.

SPECIFICITY OF HUMAN OPSONIN.

Experiments were now undertaken to determine whether or not human serum contains opsonin that is specific for pneumococci. The 24-hour surface growth of several large plain agar slants (approximately 250 sq. cm.) was washed off with 2.5 to 3 c.c. normal human serum. This mixture was then incubated from 3 to 12 hours or at room temperature for 24 hours, and then at 37° C. for two additional hours. The serum after dilution with an equal quantity of physiological salt solution was now filtered. The opsonic content of this serum was then compared with the untreated serum which was kept under the same conditions of temperature, etc. The mixtures for determining the relative degree of phagocytosis consisted of equal parts of washed normal blood, serum and salt solution, and bacterial suspension. The results obtained are presented in tabular form. (Table 1.)

[2] Bullock and Western, *Proc. Roy. Soc.*, 1906, S. B., 77, p. 531; Potter, Dittman and Bradley, *Jour. Am. Med. Assoc.*, 1906, 47, p. 1722.

TABLE 1.

SPECIFICNESS OF OPSONINS IN HUMAN SERUM.

BACTERIUM USED FOR ABSORPTION OF OPSONIN		PHAGOCYTOSIS AFTER 30 MINUTES UNDER INFLUENCE OF TREATED SERUM				
		Pneumococci "M"	Pneumococci "233"	Streptococcus "J"	Staphylococcus "C"	Tubercle Bacillus
Pneumococcus "M"		0	0	4	4.5	0.9
" "233"		0	0	3.0	2.0	0.4
" "240"	Virulent	4.2	3.5	5.6	7.0	2.0
" "241"	Virulent	4.0	1.9	6.4	5.8	1.3
" "237"	Virulent	2.5	2.1	4.8	6.0	1.4
Streptococcus C		3.0	1.4	0	3.7	1.7
Staphylococcus T		2.5	1.0	3.0	0	0.9
		PHAGOCYTOSIS UNDER INFLUENCE OF NORMAL SERUM				
		6.0	4.0	7.0	8.9	2.7

After contact with the serum for 24 hours at room temperature and for 4 hours at 37° C. the bacteria were removed by filtration.

It is shown that non-virulent pneumococci deopsonize human serum completely, so far as strains of avirulent pneumococci are concerned; at the same time they absorb some, but by no means all, of the opsonin for streptococci, staphylococci, and tubercle bacilli. Streptococci and staphylococci, on the other hand, absorb completely the opsonin for each respectively, but not all the other opsonins.

THE RELATION BETWEEN PNEUMOCOCCAL VIRULENCE AND SUSCEPTIBILITY TO PHAGOCYTOSIS AND THE POWER OF PNEUMOCOCCI TO ABSORB OPSONIN.

In the ordinary opsonic experiment the serum is allowed to act upon the bacteria for a comparatively short time—less than one hour, as a rule. As it was possible that this length of time of exposure to opsonic serum is insufficient to opsonize virulent pneumococci, a number of experiments were made in which small numbers of virulent cocci were acted upon by large quantities of sera at 37° C. for 24–48 hours, but nevertheless the cocci were not taken up by fresh leucocytes.

Human serum was treated with large quantities of pneumococci, virulent and avirulent, in order to determine whether the virulent absorb opsonin to the same degree as the avirulent. The technique employed was that just outlined, and need not be recounted here further than to say that blood agar (blood 1 part, plain agar 8–10 parts) was used to obtain large quantities of virulent pneumococci. In order to obviate the use of large amounts of blood in preparing this medium, agar was slanted in large tubes, allowed to cool, and 5 c.c. of the freshly prepared blood agar poured over the large slant and allowed to "set" before inoculation. The blood used in this way was found to have no opsonic effect upon the organisms grown on the medium.

Table 2 shows a distinct difference in the opsonic content of

TABLE 2.

ABSORPTION OF OPSONIN BY NON-VIRULENT AND VIRULENT PNEUMOCOCCI.

Strain of Pneumococcus Used in Absorption of Opsonin from Serum	Phagocytosis* (30 minutes)	
	Normal Serum	Treated Serum
Non-virulent E	25.0	0
" N	10.0	0
" N	6.0	0
" M	6.0	0
" M	4.0	0
" 233	4.0	0
" 236	6.0	0
" 236 VI	12.0	0
" R51*a*	6.4	0
" 235	5.2	0
Virulent 236 VI	10.0	0.6
" 235 I	4.0	1.0
" 236 III	3.0	0
" 240	10.0	6.0
" 240	6.0	9.0
" 241	10.0	3.0
" 241	6.0	2.2
" 241	4.0	1.9
" 237 II	6.3	5.5
" 237 IV	40.0	31.0
" 237 XI	10.0	5.5
" 237 XVIII	6.0	5.2

* The suspension of non-virulent pneumococci given in this and other tables to test the opsonic power of serum were either 24- or 48-hour cultures in plain broth. This has been found to be the most satisfactory way of procuring an even suspension of pneumococci of the proper density for routine work. Experiments proved that the presence of the broth has no appreciable influence upon opsonic action. The figures in the tables under the heading "Phagocytosis" represent the average number o bacteria per leucocyte, not less than 50 being counted at the end of 20 or 30 minutes.

serum treated with strains of non-virulent and virulent pneumococci. The non-virulent strains deopsonize the serum completely, while the virulent strains may or may not have this effect, depending upon the degree of virulency. It is interesting to note that the opsonin may be completely removed not only for the strains used in the absorption, but for other avirulent strains as well. Thus the organism R51*a* isolated four years ago deopsonized serum completely for Strain 224 which had been isolated only four weeks previously. One may therefore conclude that the specific pneumococco-opsonin is common for practically all strains of phagocytable pneumococci.

The influence of the reaction of the sera used was now studied. Table 3 shows that there is a great difference in the degree of phago-

TABLE 3.

RELATION BETWEEN THE OPSONIC POWER AND THE REACTION OF SERUM.

Sera	Reaction in Terms of *n*/40 Oxalic Acid	Phagocytosis (30 Minutes)
Fresh normal untreated serum	1.1 alkaline	38
Fresh normal untreated serum neutralized to litmus by addition of oxalic acid	Neutral	19
Alkalinity restored by adding NaOH	1.1 alkaline	28
Fresh normal serum $+CO_2$	0.5 "	28
Fresh normal serum treated with non-virulent pneumococci	0.6 "	0
Fresh normal serum treated with virulent pneumococci	0.65 "	25

NaCl solution was added to the sera to bring the dilution to the proper point.

cytosis according as the reaction differs. Neutralization with $n/40$ oxalic acid reduces it one-half. This effect is not due to destruction of opsonin because the subsequent addition of sufficient NaOH to restore the original alkalinity increases materially the phagocytosis. A reduction in the alkalinity by passing CO_2 through serum is associated with some reduction. Whether this reduction is due to destruction of opsonin, to inhibited opsonic action, or to lessened activity of the leucocytes cannot be said without further experiments. The difference in the deopsonic power of avirulent and of virulent pneumococci is not due to differences in the reaction of serum treated with non-virulent and with virulent pneumococci, because the reaction though somewhat less alkaline than normally is approximately the same in both cases. In nearly all sera treated with virulent pneumococci there is a slight reduction in the opsonic effect, which perhaps may be due to diminished alkalinity of the serum.

Much work has been given to the study of the effects of cultivation on artificial media and of passage through rabbits upon the relation of pneumococci to opsonin and opsonificability. The results obtained are illustrated in Tables 4, 5, and 6. From Table 4 we see that as pneumococcus "240" which was isolated from the blood of a case of pneumonia is cultivated upon artificial media it becomes more and more susceptible to opsonin, and, *pari passu*, its power to

TABLE 4.

INCREASE IN SUSCEPTIBILITY TO PHAGOCYTOSIS AND IN POWER TO ABSORB OPSONIN OF PNEUMOCOCCUS 240 ON CULTIVATION UPON ARTIFICIAL MEDIA.

Days upon Artificial Media	Susceptibility to Phagocytosis of Washed Pneumococcus 240 after Experiment	Susceptibility to Phagocytosis of Culture Used to Deopsonize Serum before Absorption Experiment	Phagocytosis (20 Minutes)	
			Normal Serum	Treated Serum
1	0	0	12	10.5
9	2.3	+	10	6.0
14	6.3	++	6.0	3.0
23	10.0	+++	18	4.0

TABLE 5.

SUSCEPTIBILITY TO PHAGOCYTOSIS AND POWER TO ABSORB OPSONIN OF PNEUMOCOCCUS 237 AS AFFECTED BY PASSAGE THROUGH RABBITS.

Pneumococcal Strain Used to Absorb Opsonin	Days upon Artificial Media	Susceptibility to Phagocytosis	Phagocytosis (20 Minutes)	
			Untreated Serum	Treated Serum
237	2	0	—	—
237 IV	8	+	4.5	0.6
237 VIII	2	0	6.0	4.6
237 IX	2	0	28.0	24.0
237 XI	10	+	10.0	1.5
237 XIII	2	0	6.0	4.0
237 XVIII	2	0	15.0	11.4

The Roman numerals after 237 indicate the number of rabbits through which the pneumococcus has been passed at the time of each experiment.

TABLE 6.

INCREASING RESISTANCE TO PHAGOCYTOSIS AND LOSS OF POWER TO ABSORB OPSONIN AS VIRULENCE IS INCREASED.

Pneumococcus	Days Cultivated upon Artificial Media	Phagocytosis (20 Minutes)	Remarks
236	36	38.0	
236 II	31	25.0	
236 IV	23	9.5	Complete absorption; organisms opsonified
236 VI	11	0.5	Opsonin reduced only slightly while the serum failed to opsonize pneumococcus
236 VIII	7	0	
236 VIII	7	0	
236 X	4	0	
236 XII	2	0	No appreciable reduction of opsonin; 24 hours' contact with serum failed to opsonize pneumococcus

The Roman numerals after 236 indicate the number of rabbits through which the strain has been passed at the time of the experiment.

absorb opsonin grows. Of course the results obtained on different days are not strictly comparable, although the various conditions always were kept the same as nearly as possible.

In Table 5 is shown that the susceptibility to opsonin and the power to remove opsonin from opsonic serum, of one and the same pneumococcus ("237") may be increased and diminished at will by passage through rabbits and artificial cultivation.

Table 6 illustrates a really crucial experiment of similar scope with pneumococcus "236" which, originally avirulent, was brought to a high state of virulence by successive passages through rabbits. Cultures were made on blood agar from the heart's blood of each rabbit while alive; six passages of this kind were required before the strain acquired sufficient virulence to cause prompt death of animals. The results given were obtained at the same time as the tests which were all made at the end of 36 days from the beginning of the experiment. The results again show that contact, even prolonged, of highly virulent pneumococci with serum does not result in absorption of opsonin and consequent opsonification of the bacteria. Pneumococcus 236 VI which is, so to speak, at the dividing-line between the virulent and avirulent strains is not opsonized by 48 hours' contact with serum. Marked absorption of opsonin occurs only when the organisms used are susceptible to phagocytosis, to some degree at least. The question arises whether the opsonin in such cases is absorbed only by those individual bacteria which have lost their virulence. It seems likely that this is so, because on plating out virulent pneumococci which have been cultivated for some time, colonies are obtainable of markedly different degrees of virulence.

PNEUMOCOCCAL "VIRULIN," THE SUBSTANCE UPON WHICH VIRULENCE AND RESISTANCE OF PNEUMOCOCCI TO PHAGOCYTOSIS APPEAR TO DEPEND.

In order to investigate more closely the mechanism whereby virulent pneumococci defend themselves against opsonin and phagocytosis I undertook, in accord with Dr. Hektoen's suggestion, a study of the action of pneumococcal extracts upon opsonic serum. Highly virulent and avirulent pneumococci were suspended in m/8 NaCl solution and placed at 37° C. for 48 hours. The clear fluid drawn off after thorough centrifugalization was then tested as to its action upon opsonin (Table 7). The result is quite striking. The extract from

TABLE 7.

EFFECT OF EXTRACTS OF VIRULENT AND AVIRULENT PNEUMOCOCCI UPON OPSONIC SERUM.

Equal parts of extract or NaCl and serum were incubated at 37° C. for one hour when equal volumes of washed blood and pneumococcal suspension were added.

Mixtures	Phagocytosis (20 Minutes)
Virulent extract	0.13
Avirulent extract	4.6
NaCl solution	6.0

the virulent pneumococci inhibits phagocytosis almost completely, whereas the avirulent extract does so in much less degree.

Similar results have been obtained with many extracts representing 10 strains of virulent pneumococci obtained from the blood of pneumonia patients and from post-pneumonia empyema. The extracts of five strains of non-virulent pneumococci, cultivated upon artificial media for from four weeks to four and one-half years, have given only a moderate antiopsonic effect.

The extracts are now prepared from pneumococci grown upon large blood-agar slants or in glucose-free broth to which 1 per cent dextrose is added. This broth is prepared from beef and subjected to fractional sterilization. The reaction is 1 per cent acid to phenolphthalein. Virulent pneumococci grow more abundantly and more rapidly in this medium than in the ordinary broth made from beef extract and sterilized in the autoclave. The addition of 1 volume of sterile blood to 8 or 10 volumes of broth not only makes the cocci grow better but they also yield a stronger extract.

In order to get active extracts it is necessary to suspend rather large quantities of pneumococci in relatively small amounts of salt solution. The pneumococci in about 60 c.c. of broth after 48 hours'

growth are suspended in 4 to 5 c.c. of normal salt solution and kept at 37° C. for 48 hours, heated to 60° C. for one hour, the pneumococci centrifugated down and the supernatant clear fluid, drawn off.

The conclusion seems warranted that the extract contains some substance or substances which bind or neutralize the opsonin in the serum, because active extracts do not inhibit phagocytosis by washed leucocytes of previously sensitized pneumococci. Furthermore the antiopsonic effect of virulent pneumococcal extracts is largely specific for pneumococci; as Table 8 shows the extract does not prevent the

TABLE 8.

THE SPECIFIC ANTIOPSONIC ACTION OF EXTRACTS OF VIRULENT PNEUMOCOCCUS.

Mixtures of Pneumococcal extract and Serum or NaCl Solution at 37° C. for 1 hour before adding Washed Blood and Bacterial Suspension	Phagocytosis (20 Minutes)		
	Pneumo-coccus	Strepto-coccus	Staphylo-coccus
Serum 0.1 + pneumococcal extract 0.1	0.1	7.0	10.3
Serum 0.05 + pneumococcal extract 0.15	0	3.3	9.8
Serum 0.025 + pneumococcal extract 0.175	0	1.5	5.0
Serum 0.1 + NaCl 0.1	6.0	10.6	12.0
Serum 0.05 + NaCl 0.15	5.0	6.0	10.7
Serum 0.025 + NaCl 0.175	3.2	3.0	6.0

phagocytosis of streptococci and staphylococci. After being treated in the manner described virulent pneumococci appear to become phagocytable. It must be remembered, however, that there are difficulties in the way of a clear demonstration on this point, because thoroughly extracted or autolyzed organisms are so disintegrated and stain so poorly that they are hard to see. However, if extracted organisms do become phagocytable they should absorb opsonin from serum, and, if large enough quantities are added, the pneumococco-opsonin should be removed entirely; and it has been found that when equal quantities of highly virulent pneumococci, extracted and unextracted, are suspended for 24 hours in equal amounts of serum, the extracted remove all the opsonin while the unextracted diminish only slightly the opsonic power. Unfortunately it is impossible to carry out experiments with respect to the animal virulence of the extracted pneumococci, because extraction as carried out is associated with death of the cells.

When avirulent pneumococci are suspended for 24 hours in virulent pneumococcal extract and then washed rapidly in salt solution they become relatively insusceptible to phagocytosis (Table 9) at the same time as the extract employed loses its power to neutralize opsonin (Table 10) and also becomes less toxic. This interesting

TABLE 9.

EFFECT OF VIRULENT PNEUMOCOCCUS EXTRACT UPON AVIRULENT PNEUMOCOCCI.

Equal numbers of avirulent pneumococci suspended 24 hours in the same amounts of virulent extract and NaCl sol. The cocci washed and phagocytability determined.

Extract	pneumococci	+	normal serum	+	washed blood	*aa*	2.0
NaCl	"	+	"	+	"	*aa*	25.0
Extract	"	+	serum	+	NaCl *aa* + washed blood	*aa*	6.0
NaCl	"	+	"	+	NaCl *aa* + "	*aa*	30.0

TABLE 10.

EFFECT OF AVIRULENT PNEUMOCOCCI ON PNEUMOCOCCAL EXTRACTS.

Avirulent pneumococci suspended in virulent extract 24 hours at 37° C. and then removed by filtration. The antiopsonic effect of the extract so treated compared with untreated extract:

Mixtures					Phagocytosis (20 Minutes)
Treated	extract	0.15	+ serum	0.05	2.4
Untreated	"	0.15	+ "	0.05	0
Treated	"	1	+ "	0.15	4
Untreated	"	1	+ "	0.1	0.2
NaCl solution		1	+ "	0.1	5.0

result awakened the idea that possibly the induced resistance to phagocytosis brings with it restoration of virulence. To test this possibility by experiment there was injected into the peritoneal cavity of each of three guinea-pigs of nearly the same weight the 24-hour surface growth of two blood-agar slants (approximately 20 sq. cm.) after treatment of the pneumococci (avirulent 233) in each case as follows: (1) Guinea-pig No. 1 received the pneumococci in 3 c.c. of NaCl solution in which they had been suspended for 24 hours. (2) Guinea-pig No. 2 received the pneumococci in 3 c.c. of virulent pneumococcal extract in which they had been suspended for 24 hours. (3) Guinea-pig No. 3 received the pneumococci after they had been suspended for 24 hours in virulent pneumococcal extract and then washed rapidly. The results of the detailed study of the subsequent phenomena are shown in Table 11.

The results of the examination of the peritoneal fluid and blood cultures before and after death leave no doubt that death in guinea-pigs Nos. 2 and 3 was the result of pneumococcal growth, and this growth appears to have been made possible through the acquirement of virulence by the previous treatment of the cocci in the extract. The rapidity with which the leucocytes disposed of the untreated pneumococci is especially noteworthy. No free pneumococci were found at the end of six hours, notwithstanding that such a large quantity was inoculated. In the case of the treated pneumococci the results were diametrically different. The endothelial cells which were very numerous at the end of 24 hours in the peritoneal fluid showed marked phagocytosis of polymorphonuclear leucocytes in guinea-

TABLE 11.

THE CONFERENCE OF VIRULENCE UPON AVIRULENT PNEUMOCOCCI BY TREATMENT IN EXTRACTS OF VIRULENT PNEUMOCOCCI.

Intraperitoneal inoculation of same quantity of avirulent pneumococcus after treatment for 24 hours in 3 c.c. of NaCl solution and in 3 c.c. of virulent extract.

	Guinea-pig 1 (320 grams) Pneumococci in Salt Solution	Guinea-pig 2 (325 grams) Pneumococci in Untreated Extract	Guinea-pig 3 (340 grams) Pneumococci in Untreated Exract Washed and Suspended in 3 c.c. NaCl Solution
4½ hours	Many leucocytes; phagocytosis of pneumococci marked; few free pneumococci, no endothelial cells.	Few leucocytes, many pneumococci; some phagocytosis. Seems in great pain.	Few leucocytes, some phagocytosis; no endothelial cells.
6 hours	Many leucocytes, no free pneumococci; slight phagocytosis.	More leucocytes; many pneumococci; considerable phagocytosis; seems ill.	Leucocytes abundant; many pneumococci; some phagocytosis.
24 hours	Leucocytes fairly abundant; many endothelial cells digesting leucocytes; as many as four per cell. Blood culture, negative. Seems perfectly well. Weight 310 gms.	Many leucocytes; some endothelial cells, phagocytosis of pneumococci but not leucocytes; free pneumococci abundant. Blood culture positive. Crouched, very ill. Weight 275 gms.	Very many leucocytes, phagocytic endothelial cells, phagocytosis for pneumococci but not for leucocytes, Blood culture positive.
48 hours	Leucocytes and endothelial cells few. Entirely well. Blood culture negative.	Leocucytes many; show no phagocytosis; phagocytosis of pneumococci and leucocytes by endothelial cells; free pneumococci present. Weight 240 gms. Very ill.	Less phagocytosis of pneumococci by endothelial cells; free pneumococci present. Weight 275 gms. Very ill.
72 hours	Entirely well. Weight 320 gms.	Death. Heart's blood—pure culture of pneumococci. Serofibrinous peritonitis.	Still very ill.
96 hours			Death. Findings as in Pig 2.

pig No. 1 and of pneumococci in guinea-pigs Nos. 2 and 3. It seems that the endothelial cells which appear later take up pneumococci of a higher grade of virulence than the leucocytes, for at this time the latter show no phagocytosis, even though pneumococci are present in abundance. Results similar to these have been obtained in rabbits as well as guinea-pigs with four other strains of avirulent pneumococci which had been cultivated for 3, 7, 8, and 15 months respectively. The animals receiving the pneumococci in virulent extract always showed the greater reaction, and death occurred earlier in them than in the animals which received pneumococci that had been washed after treatment in extract. That this in a measure is the result of toxic effect of the extract itself is probable, because by itself the extract is not without toxic action. It is likely also that by washing in salt solution pneumococci treated with extract a certain amount of the active substance is again extracted. Subsequent generations of the pneumococci isolated from the blood of the dead animals have virulence which increases as usual on animal passage.

The minute study of the antiopsonic body in pneumococcal extracts is now in progress. It may be stated that it resists boiling for two

minutes and that it does not appear soluble in alcohol or ether. To what extent if any it may be associated with the capsular substance of the pneumococci has not been determined. On morphological grounds there seems little reason to associate virulence of pneumococci with the capsule because it presents the same general appearance in virulent as in avirulent strains.

The chief points may be summarized as follows: It has been found possible to extract from virulent pneumococci which themselves originally do not take up pneumococco-opsonin a substance which neutralizes the opsonin in human serum; this substance unites with avirulent pneumococci and by so doing it confers upon them a degree of resistance to phagocytosis as well as of animal virulence. In other words it seems possible to extract from virulent pneumococci the substance upon which virulence would seem to depend, and at present the name "virulin," suggested by Dr. Hektoen, seems quite appropriate. While the action of "virulin" may be subject to several hypothetical explanations, at present it is probably best to look upon it simply as a substance or mixture of substances which when united with the pneumococcal cell prevent this from taking up opsonin, and which when free has special affinity for opsonin. That it does not merely concern free opsinophile cell receptors seems likely, for one reason because virulent pneumococci when extracted, i. e., freed from virulence, are found to absorb pneumococco-opsonin freely.

CONCLUSIONS.

Human serum retains its opsonic properties with respect to various bacteria after being filtered through porcelain.

The results of absorption experiments indicate that normal human serum contains several opsonins with specific affinities for pneumococci, streptococci, staphylococci, and tubercle bacilli.

Avirulent pneumococci absorb opsonin and become susceptible to phagocytosis; virulent pneumococci do not absorb opsonin and are insusceptible to phagocytosis; and these properties may be diminished or increased at will by passage through rabbits or cultivation on artificial media as the case may be.

Extraction or autolysis of virulent pneumococci in NaCl solution brings into the solution a substance or group of substances which

inhibits the action of pneumococco-opsonin; avirulent pneumococci take up this substance and now become not only resistant to phagocytosis but exhibit also to some degree the property of animal virulence; after extraction of the substance virulent pneumococci acquire the power to obsorb pneumococco-opsonin.

ISOAGGLUTINATION OF HUMAN CORPUSCLES*

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THE occurrence of isoagglutinins in human serum has been observed and studied more or less extensively by a number of investigators notably Halban,[1] Landsteiner,[2] Grünbaum,[3] Donath,[4] Descatello and Sturli,[5] Herter,[6] Ascoli,[7] Le Monaco and Panici,[8] Eisenberg,[9] Martin,[10] and others.[11]

It may be regarded as well established that the serum of the majority of healthy individuals above six years of age contains isoagglutinin; that there are several distinct isoagglutinins; that there is marked difference in the agglutinability of the corpuscles of different individuals; that autoagglutinins are very seldom if ever demonstrable *in vitro;* and that there has not been demonstrated any essential difference between isoagglutination in health and disease; hence the phenomenon at present has no diagnostic significance.

Landsteiner and, following him, Descatello and Sturli point out that individuals may be separated into three main groups by means of isoagglutination, namely: (1) those whose corpuscles are not agglutinated by the sera of Groups II and III, but whose sera agglutinate the corpuscles of these groups; (2) those whose corpuscles are agglutinated by the sera of Group III, and whose sera agglutinate the corpuscles of Group III; (3) those whose corpuscles are aggluti-

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[1] *Wien. klin. Wchnschr.*, 1900, 13, p. 545 (with Landsteiner), *Münch. med. Wchnschr.*, 1902, 49, p. 473.

[2] *Centralbl. f. Bakt.*, Orig., 1900, 27, p. 361; *Wien. klin. Wchnschr.*, 1901, 14, p. 1132; (with Leiner), *Centralbl. f. Bakt.*, Orig., 1905, 38, p. 548; (with Reich), *ibid.*, 1905, 39, p. 712.

[3] *Brit. Med. Jour.*, 1900, p. 1089.

[4] *Wien. klin. Wchnschr.*, 1900, 13, p. 497.

[5] *Münch. med. Wchnschr.*, 1902, 49, p. 1090.

[6] *Medical Record*, 1902, 61, p. 118.

[7] *Münch. med. Wchnschr.*, 1901, 48, p. 1239.

[8] *Münch. med. Wchnschr.*, 1902, 49, p. 1066.

[9] *Wien. klin. Wchnschr.*, 1901, 14, p. 1020.

[10] *Centralbl. f. Bakt.*, Orig., 1905, 39, p. 704.

[11] *Klein Wien. klin. Wchnschr.*, 1902, 49, p. 716; Schenck, *Münch. med. Wchnschr.*, 1905, 52, p. 1623; Paci, *Centralbl. f. Path.*, 1902, 13, p. 356.

nated by the sera of Group III, and whose sera agglutinate the corpuscles of Group II.

The corpuscles of Groups I and II are not agglutinated by the sera of the respective group. The corpuscles of Group III are not infrequently agglutinated by the sera of that group except the strictly homologous serum.

This general grouping, to which there are but few exceptions, is illustrated in Table 1 which gives the rearranged results of an experi-

TABLE 1.

ISOAGGLUTINATION IN TWENTY NORMAL MEN.

Corpuscles.

		I								II											III	
			4	5	12	13	14	16	19	2	1	3	6	7	8	9	10	11	18	20	15	17
SERA	I	4...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		5...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		12...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		13...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		14...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		16...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
		19...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+
	II	2...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		1...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		3...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		6...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		7...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		8...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		9...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		10...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		11...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		18...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
		20...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
	III	15...	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	+	+	+	o	+
		17...	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o

ment in which 0.1 c.c. of the serum of each of 20 normal, young adult men was mixed with 1 c.c. of a 2.5 per cent suspension of the blood of each of the 20 individuals. The readings were made after the tubes had remained in the incubator for two hours and in the ice-box over night. The exceptions to Landsteiner's grouping are given in heavy type.

This experiment has been repeated twice at intervals of one to two months with practically the same result as that given in the table. In the second test Serum 2 is recorded as not containing any agglutinin, but in the third a small amount of agglutinin for corpuscles of Group III was demonstrated. In the third test the amount of

agglutinin in Serum 13 was much smaller than on the two previous occasions, and it did not under the conditions of the experiment agglutinate all the corpuscles in Group II. The only striking and constant exception to the general scheme is the complete failure on all occasions to demonstrate any agglutinin in Serum 17. The absence of agglutinin in the sera of persons whose corpuscles fall in Group III is not at all unusual as will be made clear shortly.

In order to study still further this question of variation with respect to isoagglutination five normal persons representing the three different groups were selected and their blood tested at frequent intervals throughout a period covering several months. The results obtained have been constant with only one exception—the serum of one of the two members of Group III gradually lost its agglutinin.

Of the 76 persons whose blood has been investigated in such a manner that grouping is possible, 36 belong to Group I, 26 to Group II, and 14 to Group III. Of the 14 belonging to Group III at least six were without any agglutinin, hence we may say that about 90 per cent of individuals possess isoagglutinin (Table 2).

TABLE 2.

ISOAGGLUTINATIVE GROUPING OF SEVENTY-SIX INDIVIDUALS.

	Group I	Group II	Group III
In Health	16	14	5
Scarlet Fever	4	0	1
Pneumonia	4	3	4
Typhoid Fever	4	4	3
Advanced Pulmonary Tuberculosis	8	5	1
	36	26	14

As Table 2 shows, there is no striking alteration in the agglutinative grouping of the blood of persons suffering with various diseases, more particularly pneumonia, scarlet fever, typhoid fever, and advanced pulmonary tuberculosis. Whether any special change occurs in the original agglutinative properties of the serum or in the agglutinability of the corpuscles in the course of any of these or of other diseases has not been investigated. Of a family of five, the mother and three children belonged to Group I, the remaining child to Group II.

We may distinguish three main agglutinins, namely the agglutinin in the sera of Group I for the corpuscles of Group II and III; the agglutinin of Group II for the corpuscles of Group III; and the

agglutinin of Group III for corpuscles of Group II. That it concerns distinct bodies with special affinities for the corpuscles upon which they act is shown readily by means of absorption experiments in which definite quantities of serum are treated with an excess of corpuscles; after removal of the corpuscles by centrifugalization the agglutinative action of the serum so treated is determined in the usual way. The results are:—Corpuscles of Group I do not absorb any agglutinin; corpuscles II do not absorb agglutinin II for Group III, but they do absorb agglutinin I for both Groups II and III; and corpuscles III do not absorb agglutinin III for Group II but they do absorb agglutinin I for both Groups III and II. Of corpuscles one may say then that some have no agglutinophile receptors (Group I); that others have at least two distinct agglutinophile receptors (Groups II and III); and that corpuscles are immune to agglutinins in their own serum, if for no other reason than because of lack of suitable receptors. In addition to the three main agglutinins others no doubt occur, if only occasionally. In Group III there often is interagglutination, but I have not examined into its mechanism.

As I have pointed out elsewhere human serum may contain opsonin for human corpuscles,[1] and the question naturally arises as to what is the relation between human iso-opsonin and isoagglutinin. So far as my observations go they appear to be distinct substances, since the same serum may contain opsonin for certain corpuscles without necessarily containing agglutinin for those corpuscles, and vice versa (Tables 3 and 4). Tables 3 and 4 also show that there is marked variation in the susceptibility of the corpuscles of different individuals to iso-opsonin. I have also found that the hemo-opsonin for human

TABLE 3.

ISOAGGLUTININ AND ISO-OPSONIN IN SCARLET FEVER.

		CORPUSCLES.				
		1	2	3	4	5
SERA	1.....	**0**	**0**	**0**	**0**	+
	2.....	**0**	0	0	0	+
	3.....	**0**	0	0	0	+
	4.....	**0**	0	0	0	+
	5.....	**0**	0	0	0	0

The heavy type means phagocytosis.
0 = no agglutination.
+ = agglutination.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 721.

TABLE 4.

ISOAGGLUTININ AND ISO-OPSONIN IN TYPHOID FEVER AND PNEUMONIA.

		CORPUSCLES.							
		1	2	3	4	5	6	7	8
SERA	1....	⊡	●	●	⊡	+	+	+	+
	2....	○	○	●	○	+	+	+	+
	3....	○	○	●	○	+	+	.	+
	4....	⊡	⊡	○	○	+	+	--	+
	5....	○	●	○	○	+	+	+	+
	6....	○	●	●	⊡	+	○	+	+
	7....	○	●	●	○	+	+	○	+
	8....	○	○	○	○	○	+	○	○

1, 3, and 7 are typhoid-fever cases; 2, 8, 5, and 6 are pneumonia cases; 4 is serum disease. For explanation of signs see note under Table 3.

corpuscles in the serum of normal or diseased human beings is not absorbed by corpuscles that resist its opsonic action. On the contrary corpuscles that are subjected to phagocytosis, when treated with opsonic serum, remove opsonin from the serum. In this respect there is complete analogy with the relation between non-agglutinable and agglutinable corpuscles to isoagglutinin. The experiments are not yet extensive enough, however, to determine anything definite as regards the kinds of opsonin in human serum.

It has not been possible to demonstrate the presence in human serum or citrated plasma of any definite and pronounced anti-isoagglutinin. The admixture of increasing quantities of sera from individuals of one group to sera of members of either of the other groups does not in any way hinder in any decisive manner the clumping of the proper corpuscles. Agglutination takes place in mixtures of defibrinated blood, containing isoagglutinins and agglutinable corpuscles. Again, agglutination takes place in a mixture of the freshly drawn blood of an individual of Group I with an equal quantity of that of a member of Group III, coagulation being prevented by adding as much again as the total quantity of blood of citrate solution (1 per cent of citrate of sodium in m/8 NaCl solution) or by means of hirudin solution. On account of the great mass of blood corpuscles in such mixtures the occurrence of agglutination may be recognizable under the microscope only.

The prolonged treatment of erythrocytes with serum free from suitable isoagglutinin in no way hinders or delays the agglutination of the corpuscles by active serum.

Human isoagglutinins are stable bodies—heating serum to 60°

C. for 30 minutes does not destroy them and they persist in serum kept in the ice-box for many months, even when contaminated with bacteria. Serum filtered through porcelain retains its original agglutinating power practically unchanged.

The amount of agglutinin varies considerably in sera from different persons (Table 5). Several comparative demonstrations by means of

TABLE 5.

THE SMALLEST AMOUNT OF VARIOUS SERA CAUSING DISTINCT ISOAGGLUTINATION.

5 per Cent Suspension of Corpuscles+Serum in NaCl Solution—each 0.5 c.c.

SERA (SOURCE AND GROUPING)	GROUPING OF CORPUSCLES			
	2	2	3	3
Normal..... 1	.0015	.006	.012	.006
Normal..... 2	....	....	.006	.003
Normal..... 2	....	....	.025	.012
Normal..... 3	.0015	.0015	....	....
Pneumonia..... 1	.0015	.0015	.006	.006
Typhoid fever..... 1	.003	.006	.006	.025
Typhoid fever..... 1	.006	.006	.025	.025
Typhoid fever..... 3	.012	.006	....	....
Scarlet fever..... 1	.006	.006	.025	.025
Chronic pulmonary tuberculosis.. 1	.006	.003	.006	.003
Chronic pulmonary tuberculosis.. 2	....	....	.006	.012
Chronic pulmonary tuberculosis.. 3	.012	.006	....	....
Serum disease..... 1	.0003	.0003	.0003	.0003
Serum diseas 2	....	....	.003	.003

progressive dilutions of the sera have failed so far to reveal any larger amount of isoagglutinins in the blood of patients with scarlet fever, pneumonia, typhoid fever, and tuberculosis than that of normal persons, but further observations are necessary before final conclusions are justifiable. In the sera of two cases of serum disease, one of which belonged to Group I, the other to Group II, the amount of isoagglutinin was the greatest of any serum yet examined; .0003 c.c. of the serum of the case in Group I being strongly agglutinative. Table 5 also shows that the different corpuscles of the same group vary in their agglutinability by the same serum; furthermore that sera of Group I may contain different amounts of agglutinin for Groups II and III.

I have not been able to find any isoagglutinins in the serum of rabbits, guinea-pigs, dogs, horses, and cattle. In each instance 0.1 c.c. of 10 or 20 different sera were tested upon 1 c.c. of a 2.5 per cent suspension of washed corpuscles from as many animals.

From the practical point of view isoagglutination of human corpuscles is of immediate interest in connection with the determination

of the opsonic index and with transfusion of blood. All who have worked with the opsonic index are familiar with the fact that occasionally the mixtures show marked clumping of the erythrocytes which naturally interferes more or less with the desired accuracy. In order to avoid this annoyance the washed blood (leucocytes) should be secured from an individual of Group I, i. e., from one whose corpuscles are not agglutinable.

By means of a delicate and highly refined technique Crile[1] recently has practiced direct transfusion of blood from normal to diseased human beings, and with marked apparent success so that it is not unlikely that the practice of transfusion may increase. The common occurrence of isoagglutinins in human serum suggests that under certain special conditions homologous transfusion might prove dangerous by leading to erythrocytic agglutination within the vessels of the subject transfused. It has not been feasible to conduct animal experiments upon this point because of failure to demonstrate isoagglutinins in the serum of suitable animals. It may be pointed out, however, that the possible danger here indicated can be avoided by the selection of a donor whose corpuscles are not agglutinated by the serum of the recipient, and whose serum does not agglutinate the corpuscles of the latter; that is to say, donor and recipient should belong to the same group and preferably to Group I or II. The actual relation *in vitro* of the sera of prospective donors and the recipient to the respective corpuscles are readily determinable in a short time in the manner outlined in the foregoing.

[1] *Jour. Am. Med. Assoc.*, 1906, 47, p. 482.

THE STREPTOCOCCO-OPSONIC INDEX IN SCARLATINA.*

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ON account of the close association of streptococci with scarlatina, it was thought that it would be of interest to study the streptococco-opsonic power of the blood throughout the course of this disease.

TECHNIQUE.

The Leishman-Wright method of estimating the opsonic index has been employed in these examinations: Equal volumes of serum, washed corpuscles, and streptococcal suspension are thoroughly mixed in a capillary pipette and the mixtures incubated at 37° C. for 15 minutes, when smears are made and stained. The streptococcal suspension is made from a 24-hour culture on plain agar, or horse-serum agar if the organism does not grow well on the plain agar. The fluid of condensation is discarded because of the clumps and long chains of cocci that it usually contains. The growth on the solid medium is washed off with enough 0.85 per cent salt solution to produce a slightly opalescent fluid. An effort is made to obtain a suspension of sufficient thickness to give an average count of five organisms per leucocyte in the normal specimen.

The sera from three normal individuals is pooled and used as the normal control. The pool is collected the same day as the patient's serum, but both may be kept 24 hours before the index is determined. Blood from the same individuals is generally employed for the pool, and the washed blood (leucocytes) obtained from an individual whose erythrocytes are not susceptible to isoagglutination. Fifty polymorphonuclear neutrophiles are counted, 25 at each end of the slide. If these two counts do not correspond closely, more leucocytes are counted. If the suspension is thin, so that there is an average of 1–3 organisms per leucocyte, 100 cells are counted. When the suspension is thin, the percentage of phagocyting cells is estimated. Generally this percentage index corresponds closely to the opsonic index. Clumps of leucocytes are disregarded.

OBSERVATIONS ON STREPTOCOCCO-OPSONIN

It is now well known that normal human serum as well as the serum of many animals contains opsonin for various strains of streptococci.

In a large number of preliminary tests I found that the streptococco-opsonic index of normal adult persons may vary between 0.90 and 1.1. In these experiments as well as in the earlier experiments with the sera of scarlet fever patients a typical *Strept. pyogenes*, isolated by Dr. Ruediger from a case of otitis media in scarlatina and which ferments mannit, was employed by preference, not only on account

* Received for publication April 1, 1907.

of its source but also because of its relatively uniform suspensions. In order to determine to what extent the opsonic index in case of this particular strain ("Laura") could be regarded as true for other similar strains, a number of comparative tests were made. As shown in Table 1, only slight variations were detected, and most of the

TABLE 1.

COMPARATIVE STREPTOCOCCO-OPSONIC INDICES OBTAINED WITH STREPTOCOCCUS "LAURA" AND HOMOLOGOUS STREPTOCOCCI.

SERUM "A"		SERUM "B"		SERUM "C"		SERUM "D"		SERUM "E"	
Strept. "Laura"	Strept. "A"	Strept. "Laura"	Strept. "B"	Strept. "Laura"	Strept. "C"	Strept. "Laura"	Strept. "D"	Strept. "Laura"	Strept. "E"
1.34	1.7	1.7*	0.77*	1.4	1.0	1.4	1.2	1.24	1.4
1.0	1.0	1.3	1.7	1.3	1.28	0.89	0.99	1.0	0.9
0.94	0.94	0.98	1.2	0.97	1.0	1.0	1.1		
1.26	1.28	1.2	1.5	0.91	0.89				
0.9	0.9	1.2	1.1	1.09	1.1				
1.28	1.2								
1.14	1.0								
1.18	1.2								

Serum "A" from scarlet-fever patient with cervical abscess from pus of which streptococcus "A" was isolated.

Serum "B" from scarlet-fever patient with otitis media from pus of which streptococcus "B" was isolated.

Serum "C" from erysipelas patient; streptococcus "C" isolated from abscess in leg.

Serum "D" from scarlet-fever patient; streptococcus "D" isolated from throat.

Serum "E" from scarlet-fever patient; streptococcus "E" from throat.

* No evident explanation at hand for this divergence.

observations recorded here have been carried on with streptococcus "Laura" controlled, however, from time to time by the use of streptococcal strains isolated from the very patients whose sera were being studied.

It was considered possible that heating the normal and scarlatinal sera might bring out greater differences in the opsonic indices. While this proved to be the case in occasional instances (Table 2),

TABLE 2.

STREPTOCOCCO-OPSONIC INDICES OF HEATED AND UNHEATED SCARLATINAL SERUM.

Unheated	Heated	
1.43	1.60	
1.30	1.30	
1.30	1.20	
1.00	2.50	
1.20	1.90	55° C. one hour
0.96	0.99	
0.96	1.00	
0.97	0.94	
0.86	0.84	
1.60	1.30	
1.40	1.30	60° C. 30 min.
1.36	1.70	

the indices in general were found to remain about the same as when unheated sera were used. Heating human serum (normal and scarlatinal) to 55° C. for one hour reduces the streptococco-opsonic power very materially; heating to 60° C. for half an hour reduces the opsonin content to a fraction of the normal.

Dilutions of serum with 0.85 per cent NaCl solution gave about the same indices as the undiluted serum when the relation of serum to solution was as 1:10 and 1:20; in higher solutions in some cases the scarlatinal sera gave very high indices, but for the present purpose it was thought best to follow the usual methods.

The specificity of streptococco-opsonin in scarlet fever.—During the course of this investigation the opsonic index of scarlet-fever patients with respect to staphylococci, pneumococci, *Strept. viridans* and pseudodiphtheria bacilli has been determined repeatedly. The results may be summarized in the statements that the opsonic index for staphylococci, pneumococci, and *Strept. viridans* was found to be normal on all occasions while that for *Strept. pyogenes* varied from 0.5–2.6; nor was there any correspondence in two cases examined between the streptococco-opsonic index and the index for the pseudodiphtheria bacilli isolated from the suppurating ears in these instances. The streptococco-opsonic index of uncomplicated cases of measles and of typical pneumonia was found to range wholly within normal limits (0.96–1.1). In erysipelas, however, the streptococco-opsonic index was found to present a sharp rise at about the time the temperature began to fall. In diphtheria there is also a rise in the streptococco-opsonic index early in the disease. These facts seem to me to indicate that the streptococco-opsonin in scarlet fever is a specific opsonin.

THE STREPTOCOCCO-OPSONIC INDEX IN SCARLET FEVER.

The blood of 16 scarlatinal patients has been examined, generally daily during the acute stage and at greater intervals during convalescence. In 10 cases during the acute stage the opsonic index was found below 0.9, ranging from 0.5 to 0.88; in five between 0.9 and 1.0; and in one only not below 1.0. As I found the normal opsonic index to streptococci to vary between 0.90 and 1.1, the cases with indices of 0.9 can scarcely be considered outside the normal limit.

With the fall in temperature and improvement in the patient's

symptoms there occurs a rise in the index to above normal; this was true of all my cases except in a quickly fatal case in which the index did not rise above 1.0. In seven cases, all complicated, the opsonic index rose above normal while the fever was still high, but at this time there seemed in each case to be an improvement in the general condition.

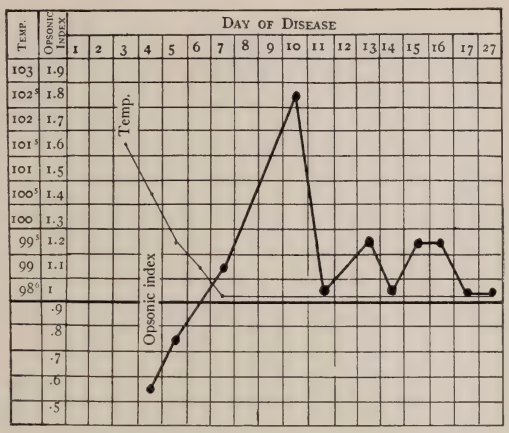

CHART 1.—Mild, uncomplicated scarlet fever. (Male, age 16.)

This usual rise in the index is succeeded by a fall to normal, often more or less abrupt, as may be seen in Charts 1, 2, 3, as well as others. The index remains at or near normal during convalescence in less severe, uncomplicated cases. Chart 4 shows the fluctuations of the index in a severe case without evident special complications.

The cases (six) in which the opsonic index at first examination was not below normal were not observed until on the 5th–7th day, and at this time the index may have recovered from the initial fall. In two of these cases diphtheria coexisted with scarlet fever, but without any special effect upon the course of what may be regarded as the typical streptococcus-index of scarlet fever.

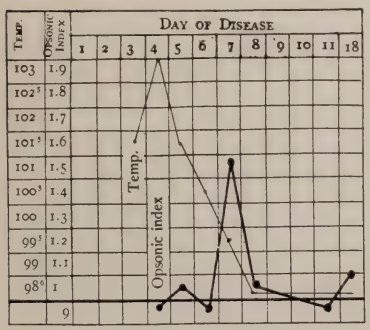

CHART 2.—Mild, uncomplicated scarlet fever. (Adult female)

In the cases that were free from special complications during the early part of the attack, the rise of the index above normal occurred between the 5th and 11th days; most often between the 7th

and 9th. According to the observations of Bowie[1], Klotz,[2] Tileston and Locke,[3] this is also about the time when the leucocytosis of scarlet fever is at its height. The polymorphonuclears are now relatively increased. This coincidence of the streptococco-opsonic curve and the leucocytosis may be regarded as marking the high level of the streptococcidal powers of the blood in scarlet fever.

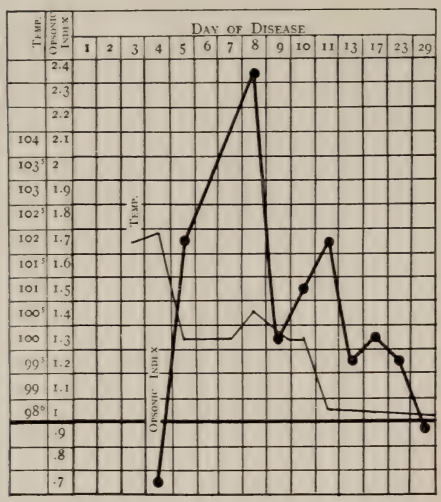

CHART 3.—Mild, uncomplicated scarlet fever. (Male, age 10.)

In the cases in which the indices showed a tendency to remain above normal for some time after the primary rise there occurred no streptococcal complications. Definite local streptococcal complications appear to be

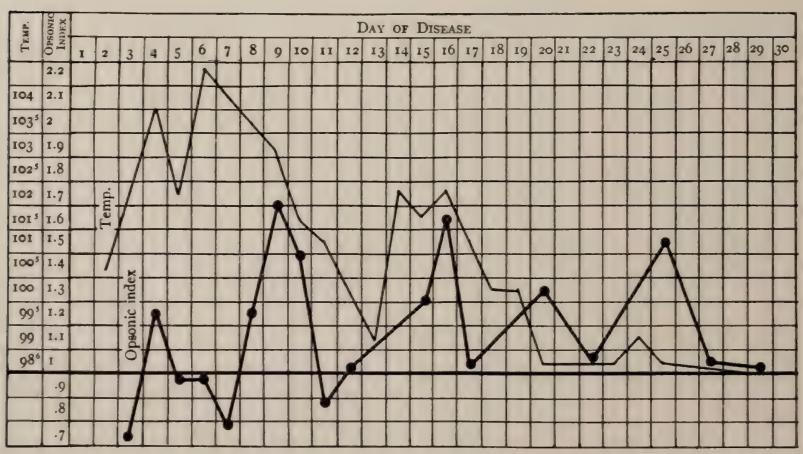

CHART 4.—Severe scarlet fever. (Female, age 12, no special complications; recovery.)

[1] *Jour. Path. and Bact.*, 1902, 8, p. 82.
[2] *Jour. Infect. Dis.*, 1904, 1, p. 404.
[3] *Ibid.*, 1906, 3, p. 375

initiated with low streptococco-opsonic indices; as improvement takes place, the indices commonly rise (Charts 5, 6, 7). Although no cultures were made from the joints in the case illustrated on Chart 5, the course of the index indicates the streptococcal nature of the articular lesion.

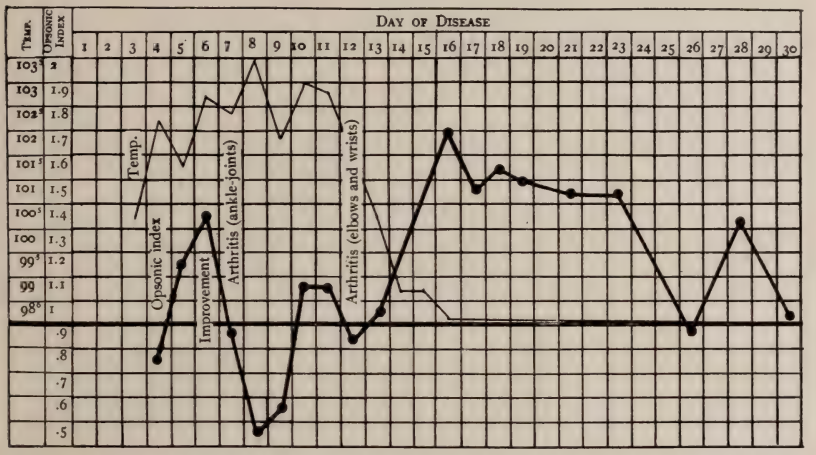

CHART 5.—Severe scarlet fever with joint complications. (Male, age 13; recovery.)

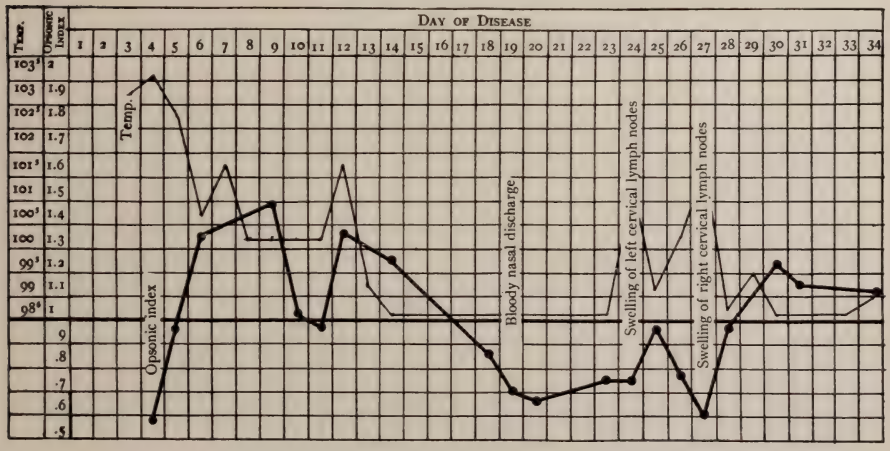

CHART 6.—Severe scarlet fever with cervical lymphatic involvement. (Male, age 14; spontaneous resolution; recovery.)

In two cases of postscarlatinal streptococcal otitis (not included in the 16 cases discussed here) the indices rose above normal as the

discharge diminished. In otitis media due to other bacteria, e. g., pseudodiphtheria bacilli, the streptococcal index did not vary in any such accord with the clinical course of the infection.

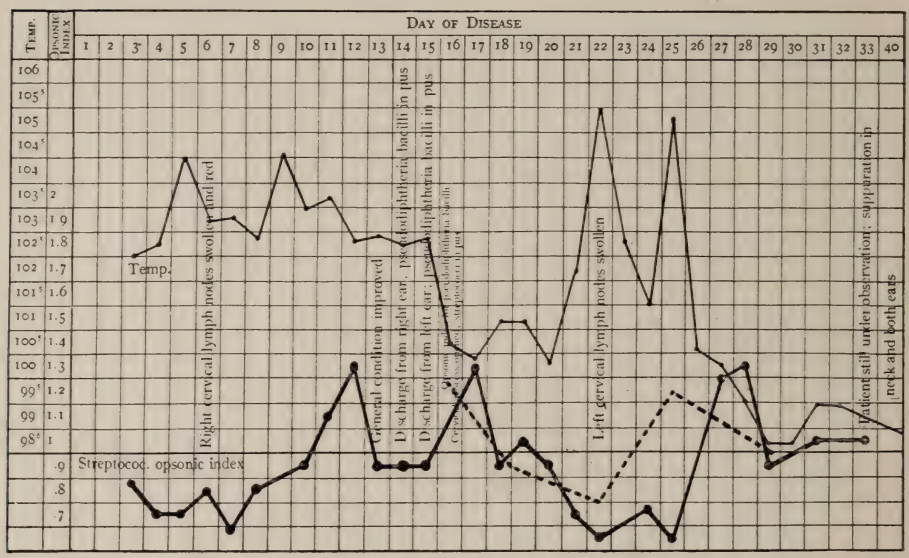

CHART 7.—Scarlet fever with suppuration in cervical lymph nodes and ears. (Female, age 5; recovery.)

In a fatal case complicated by broncho-pneumonia, the streptococco-opsonic index gave a sharp primary rise (Chart 8) with an abrupt fall coincidently with the onset of the pneumonia. During the rest of the illness the index remained about normal. A few days before death, cultures of the blood gave streptococci in pure growth. The cause of the pneumonia was not determined and there was no autopsy. Judging from Chart 8 there was after the 11th day of the prolonged illness in this case no marked reaction of the organism to streptococcal products. Whether this should be interpreted as meaning that the streptococcus played no special part in the subsequent events or was the result of failure of response on the part of the organism, must be left unsettled.

It may be of interest to mention again that in diphtheria there occurs a sharp rise in the streptococco-opsonic index somewhat earlier

in the disease than seems to be the case in scarlet fever and, so far, the streptococcal index has not been found below normal in the acute

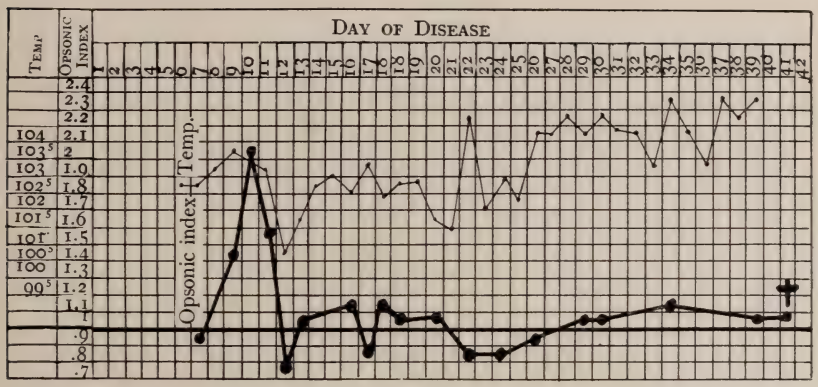

CHART 8.—Scarlet fever, broncho-pneumonia; death. (Female, age 15; broncho-pneumonia developed about 11th day; no tubercle bacilli in sputum; tuberculo-opsonin index at one time .7; terminal streptococcemia.)

stage of diphtheria. The index for diphtheria bacilli in diphtheria is low at the outset, rising above normal quite considerably as the membrane disappears and the symptoms abate.

THE RELATION OF STREPTOCOCCI TO SCARLET FEVER AND CONCLUSIONS.

The results of this study are fully in accord with the view that streptococci play an important part in scarlet fever. The variations in the streptococco-opsonic index even in mild cases, show that practically from the first the scarlet-fever patient is the subject of a definite streptococcus infection. The course of the index indicates that this infection runs parallel with the phenomena of the acute stage of the attack and hence must be held responsible for at least some of these phenomena.

There is no question concerning the streptococcal nature of most of the complications of scarlet fever and as would be expected such complications influence the streptococco-opsonic index accordingly, there being an initial fall succeeded by a definite rise as the infection subsides. The conditions in the severe, so-called "septic" cases merit further study. Investigations are also under way for the pur-

pose of determining the preventive and therapeutic effects of streptococcus vaccine in scarlet fever and its complications.

At present the following conclusions seem warranted:

In the beginning of scarlet fever the streptococco-opsonic index in the majority of cases is below normal. As the acute symptoms subside the index rises above normal to which it soon returns, sometimes quite abruptly. In uncomplicated cases it commonly remains practically normal during convalescence. Definite local streptococcal complications are inaugurated by a depression in the streptococco-opsonic index which rises again as improvement takes place.

THE OPSONIC INDEX AND VACCINE THERAPY OF PSEUDODIPHTHERIC OTITIS.*

ALICE HAMILTON.

(From the Memorial Institute for Infectious Diseases, Chicago.)

ALMOST a year and a half ago, in the course of experiments made by Dr. Horton and myself to determine the nature of a certain immune serum, it was found that opsonins for pseudodiphtheria bacilli are present in human blood and in the blood of some of the lower animals, and that these bodies can be increased by repeated injections of cultures of the bacilli.[1] The pseudodiphtheria strains used in these experiments belong to the variety usually known as the "Ruediger bacillus," and are distinguished from other varieties by the fact that they are virulent for guinea-pigs and that the serum of rabbits immunized against them is bacteriolytic for this variety but not for other pseudodiphtheria bacilli. The serum of human beings was found to contain opsonin for these bacilli and also the serum of the dog, goat, rabbit, and guinea-pig. Rabbits and goats were immunized by injections of broth cultures of one of the strains, and the resulting serum, which was strongly bacteriolytic and agglutinative, had also increased greatly in opsonic content. "Nine experiments were made with leucocytes of lower animals and 40 with human leucocytes. Without exception, in all of these experiments the presence of our immune serum favored phagocytosis of this group of bacilli, but had no effect on phagocytosis of *B. diphtheriae*, or of six strains of ordinary pseudodiphtheria bacilli. There was practically very little phagocytosis in the tubes containing no serum, there was a varying amount in the tubes with homologous serum, but in the experiments with the virulent pseudodiphtheria bacilli there was always a marked increase in the amount of phagocytosis in the tubes with immune serum as compared with normal rabbit serum." In these experiments the serum, washed corpuscles, and bacterial suspension were used in the

* Received for publication April 6, 1907.

[1] Hamilton and Horton, *Jour. Inject. Dis.*, 1906, 3, p. 128.

proportion of one part each of the first two to two parts of the last. As a thick bacterial supsension was used and the tubes incubated for 45 minutes, the number of bacteria taken up was large. The results were stated in numbers representing the average number of bacilli ingested by 40 leucocytes. Normal rabbit serum being taken as the control, we should have an index of 4.9 for the immune rabbit serum when used with human leucocytes. The opsonin in the immune serum was specific for these bacilli, *B. diphtheriae* and the ordinary varieties of pseudodiphtheria bacilli were not affected by it, the index for the former being 0.85 and for the latter (an average of eight counts) 0.8. Immune goat serum also contained increased opsonin for the Ruediger bacillus but not for other varieties of pseudodiphtheria bacilli nor for *B. diphtheriae.*

To carry on similar experiments with the serum of human beings instead of serum of the lower animals it would be necessary to have under observation cases in which the Ruediger bacillus had been found, but none was at hand. Instead was studied a class of patients which were easily available, namely patients suffering from a form of otitis media (see p. 326 of this number) in which pseudodiphtheria bacilli are found in large quantities. As pointed out in the following article, these bacilli resemble the Ruediger bacillus in many ways, but differ from it in that, while most strains of the latter are non-fermenters, the former ferment dextrose, some of them ferment saccharose (Group 1), and those which do not ferment saccharose ferment maltose (Group 2). The chief distinction, however, lies in the fact that the serum of rabbits immunized against the Ruediger bacillus contains bacteriolysin, agglutinin, and opsonin for the Ruediger bacillus, but acts simply like normal rabbit serum toward both types of the pseudodiphtheria bacilli of otitis media, while conversely the serum of a rabbit immunized against Group 1 of the latter contains bacteriolysin, agglutinin, and opsonin for the active strains of this group, but not for the Ruediger bacillus.

The results of the opsonin tests made with the immune Ruediger serum have been given. The following are the indices obtained with the serum of a rabbit immunized against a strain of Group 1 (fermenting saccharose). The serum of normal rabbits was used as control.

Group 1	4.0
Group 1	2.2
Group 1	2.1
Group 2 (fermenting maltose)	0.8
Group 2	1.1
Ruediger Bacilllus	0.9

The Ruediger bacillus was found only three times in an examination of 142 cases of otitis media, but the two other varieties were found 51 times. Seventy-two per cent of the cases of scarlatinal otitis media contained them. The arguments as to the etiological significance of this organism are given elsewhere, and it is only necessary to state here that the results of the opsonic tests made with serum from these patients go to strengthen the belief that the bacilli in question are the causative agents in certain cases of otitis media.

Twenty-two cases of otitis media from which the pseudodiphtheria bacillus had been isolated were selected and their opsonic index to their own strain was determined. Seventeen were scarlet-fever patients, two measles, and three were cases of chronic recurrent otitis, who were at the time suffering from an attack of the disease. As control, seven cases of scarlet fever and measles were taken, two of which had otitis media, but the pus in these cases yielded streptococci only. Finally 16 examinations of serum from normal persons were made. For these control tests, five of the strains of pseudodiphtheria bacilli isolated from otitis media cases were used, three belonging to Group 1, two to Group 2.

Table 1 gives the indices obtained from the normal cases, from the measles and scarlet-fever cases without pseudodiphtheria infection, and from the cases with pseudodiphtheria bacilli in the pus from the ear. Only two of the pseudodiphtheria cases had indices within the normal limits, the others were all very much below. In two of the cases in this table it was possible to make but one test, in eight more, from two to five, could be made, and in the remaining 12 the examination could be carried on for a period covering several weeks. The index of these patients was found to have quite a wide range and a certain correspondence between the clinical symptoms and the index could be noted. Thus one case, a chronic recurrent purulent otitis, had an index of 0.3 during an attack and of 1.2 at the time of discharge. Another chronic case had an index of 0.4 at the beginning and of 1.3 at the end of the attack. A case of acute

scarlatinal otitis, in which the index was taken three times, began with 0.8, fell to 0.2, and was up to 1.0 at the end. As would be expected

TABLE 1.

THE OPSONIC INDEX FOR PSEUDODIPHTHERIA BACILLI IN PSEUDODIPHTHERIC OTITIS, MEASLES, AND SCARLET FEVER, AND IN HEALTH.

Otitis Media	Measles and Scarlet Fever	Normal Persons
0.11 (scarlatinal)	0.7	0.6
0.11 "	0.9	0.7
0.17 "	0.9 streptococcal ear	0.8
0.19 "	0.9 " "	0.81
0.19 "	1.0	0.9
0.2 "	1.0	0.9
0.2 "	1.16	1.0
0.2 "		1.0
0.26 (measles)	0.95	1.0
0.3 (scarlatinal)	Average	1.09
0.3 "		1.1
0.3 "		1.1
0.3 (chronic recurrent)		1.1
0.38 (measles)		1.25
0.38 (scarlatinal)		1.3
0.38 "		1.3
0.4 (acute non-scarlatinal)		
0.4 (scarlatinal)		0.99
0.43 "		Average
0.46 "		
0.7 "		
1.3 (acute non-scarlatinal)		
0.35 Average		

the indices of the patients showed a much wider range than those of normal individuals. The following are the lowest and highest indices in four cases:

Lowest	Highest
0.2	2.1
0.11	1.6
0.19	2.4
0.39	2.0

Those cases, which were followed for several weeks, showed sometimes a persistently low index and sometimes one with variations corresponding to the subsidence or exacerbation of the symptoms. Two instances of low index are given in Charts 1 and 2. The case illustrated in Chart 1 had a profuse suppuration which persisted up to the time of dismissal from the hospital. The case illustrated in Chart 2 was not nearly so severe a case and there was a decided improvement in the condition of the ear before she left the hospital. In Charts 3 and 4 there is more variation in the indices and the clinical notes show that the index rose or fell before the change in the condition which the

rise or fall foreshadowed. Thus in the case illustrated in Chart 3 there was a marked improvement in the condition coincident with the

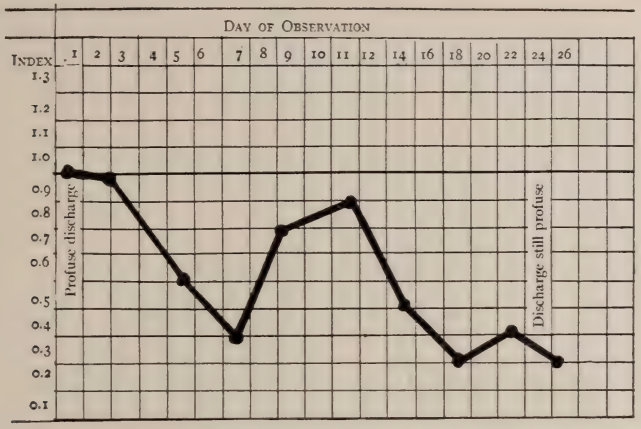

CHART 1.—The opsonic index for pseudodiphtheria bacillus (Group 1) in acute otitis.

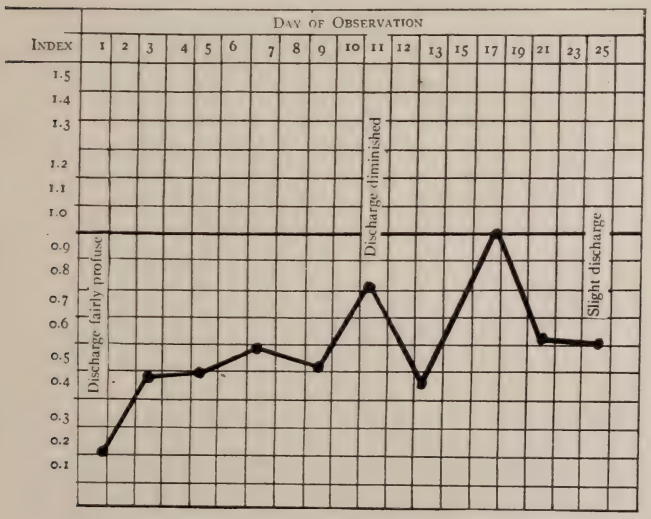

CHART 2.—The opsonic index for pseudodiphtheria bacillus (Group 1) in acute otitis.

higher index. When the blood was taken on the 24th day it was supposed that the index would prove normal, and the drop here was not explained until, on the 27th, the ear was found to be discharging

profusely again. Chart 4 is that of a child who began with a serious suppuration, improved, and then relapsed and was dismissed with a low index, the ear still suppurating. On the other hand, Chart 5

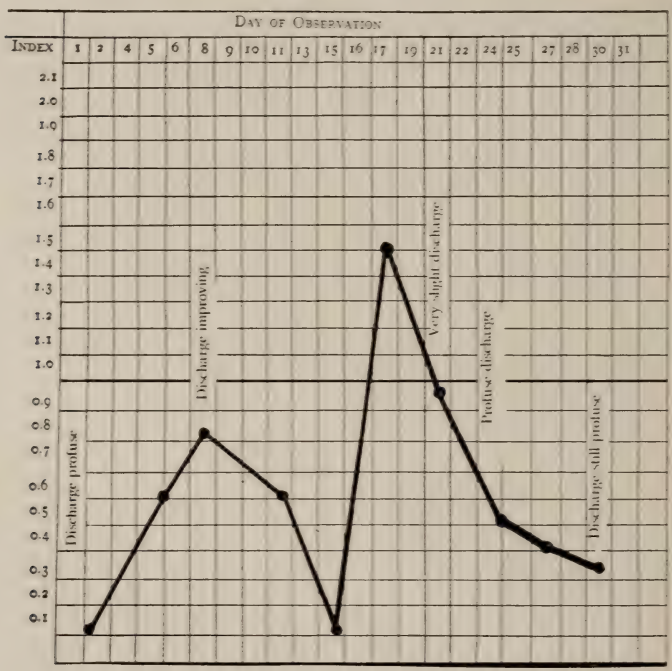

CHART 3.—The opsonic index for pseudodiphtheria bacillus (Group 2) in acute otitis.

shows a curve with great variations and yet the course of the disease was fairly constant, the suppuration was always quite profuse. When the child left the hospital the index was very high, but the ear was not entirely well.

The study of these indices affords a strong argument in favor of the pyogenic character of the pseudodiphtheria bacillus. Those cases of otitis media which yielded cultures of pseudodiphtheria bacilli had—with the exception of two cases in each of which but one examination was made—very low indices to these bacilli. The cases of measles and scarlet fever which had no suppuration in the ear or in which there was a streptococcal infection of the ear, had indices to the pseudodiphtheria bacilli which averaged those of the

normal persons examined—0.95 and 0.99. The fact that the variations in the index of these cases corresponded often to the variations in the clinical symptoms also points to the fact that the pseudodiphtheria bacilli was the cause of the suppuration.

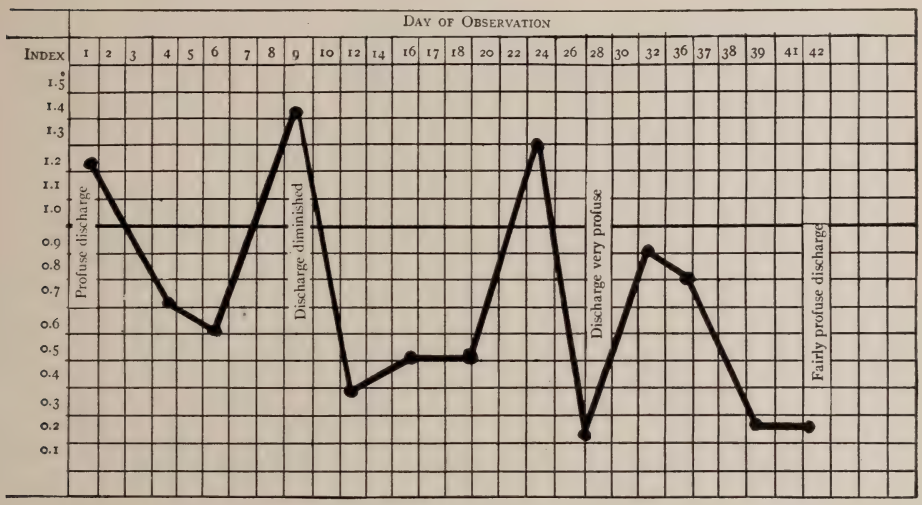

CHART 4.—The opsonic index for pseudodiphtheria bacillus (Group 1) in acute otitis.

The opsonin for these bacilli appears to be specific. Patients suffering with streptococcal complications were selected and their index to the streptococcus and to the pseudodiphtheria bacillus taken with the following result:

	Streptococcus	Pseudodiphtheria bacilli
J. P.	1.4	0.98
M. C.	0.5	0.9
K. G.	1.3	0.29 (Pseudodiphtheric otitis)
A. M.	0.9	0.11 (Pseudodiphtheric otitis)

In one instance the streptococcus index was followed for a long period by Dr. Tunnicliff and the pseudodiphtheria index by myself, the child having a streptococcal suppuration in the cervical glands and pseudodiphtheria bacilli in the pus from the ear. The two curves when superimposed (Chart 6) show that a high index for one organism did not mean a high one for the other, a circumstance that indicates two kinds of opsonin.

As it had been shown that injections of the Ruediger bacillus

increases the opsonin in the serum of rabbits and goats, it seemed probable that an analogous phenomenon could be produced in human beings by injections of the bacillus isolated from the suppurating ears, and that the condition might possibly be improved by this increase of the specific opsonin for these bacilli. Seven cases of profuse suppurative scarlatinal otitis media were selected for the study of this point. One child had had discharge for nine weeks, the other 6 for periods varying from 3 to 10 days. It would have been very much more satisfactory to have carried out these experiments with old, chronic, and obstinate cases which had not yielded to ordinary methods of treatment, for in such cases it would be comparatively easy to show whether or not vaccine therapy had any value. In acute scarlatinal otitis media we are dealing with a process which is subject to sudden, spontaneous cure, so that it is practically impossible to demonstrate that any remedial agent influences its course. But the difficulty of controlling dispensary patients, the impossibility of making as many and as frequent tests as would be necessary, forced me to turn to the material in the hospital and to take acute instead of chronic cases.

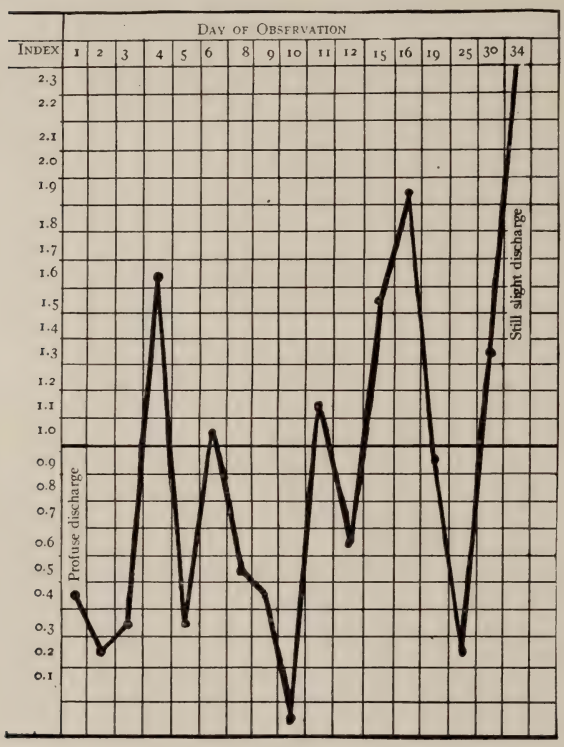

CHART 5.—The opsonic index for pseudodiphtheria bacillus (Group 2) in acute otitis.

The vaccine for these injections was prepared by suspending in

normal salt solution a twenty-four-hour old agar-slant culture and heating it to 65° for forty minutes. In all cases the absolute sterility of the vaccine was demonstrated by cultural methods. The dose given at first was one-half of this amount, then it was increased to three-quarters and then to a whole culture, but as the effect of the

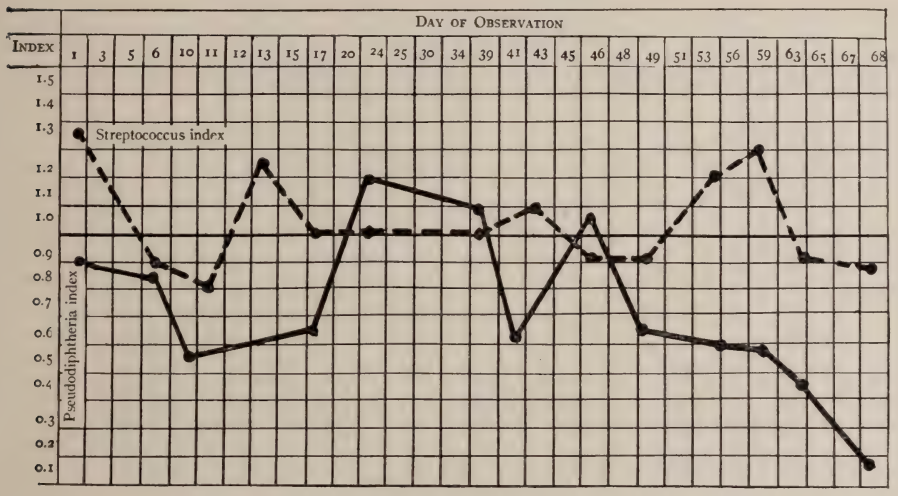

CHART 6.—The opsonic indices for pseudodiphtheria bacillus (Group 1) and staphylococcus in pseudodiphtheric otitis and cervical streptococcal infection.

larger doses was no greater than that of the small it seemed best to return to the original dose and one-half a slant culture was given regularly. One case had a bacillus of Type 2, growing rather scantily on agar and the dose in this instance was a full slant culture.

No local reaction followed the injection of these vaccines and no constitutional reaction, so far as could be discovered. The first case had to be abandoned after two injections because the child developed laryngeal diphtheria and a very weak heart which made it inadvisable to persist in an experimental treatment. The first injection had raised the index from 0.25 to 1.6, the second from 0.5 to 1.15. No change was noticed in the condition of the ear.

The second case was given two injections and then was dismissed from the hospital. The suppuration was still fairly profuse, the index had risen only to 1.15 and at dismissal was 0.7. The third

case was more satisfactory. This child had had a profuse suppuration of the middle ear for nine weeks. She was given two injections, the treatment extending over 13 days. At the end of that time she was

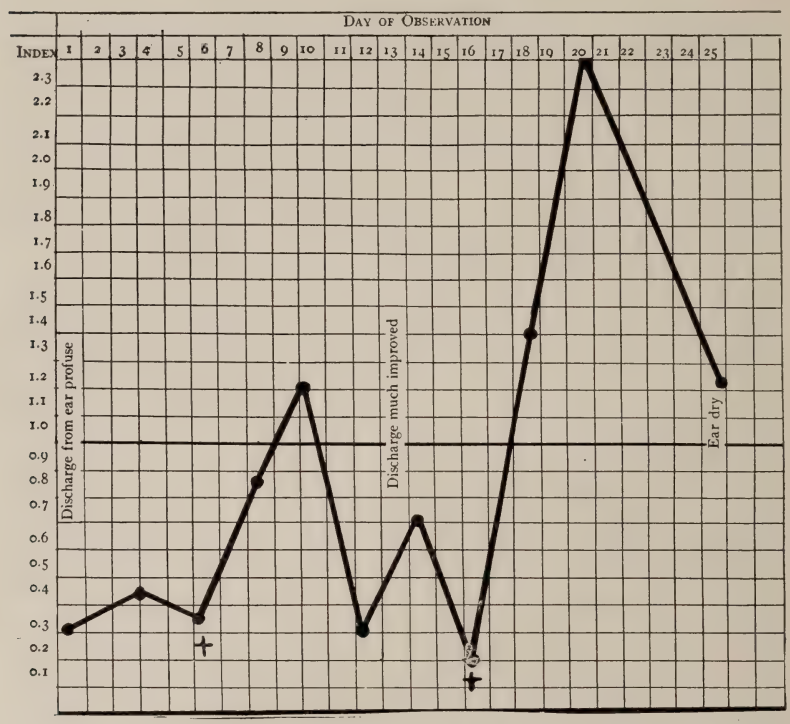

CHART 7.—The opsonic index in pseudodiphtheric (Group 1) otitis treated with autogenous vaccine. + = injection of vaccine.

dismissed with only a slight, thin discharge from the ear. Her index rose from 0.3 to 1.3 after the first injection. The charts of the other four are given (Charts 7, 8, 9, and 10). It will be seen that in all, the injections were followed by a more or less pronounced rise of the patient's index, often without any preliminary negative phase. In all four cases the clinical symptoms also improved and two of the patients (Charts 7 and 10) had no discharge when they left the hospital, the other two (Charts 8 and 9) had a slight discharge. However, at the same time and in the same two wards four other children, all with the same form of otitis media, made as rapid and as good recovery as these four, without any injections of vaccine.

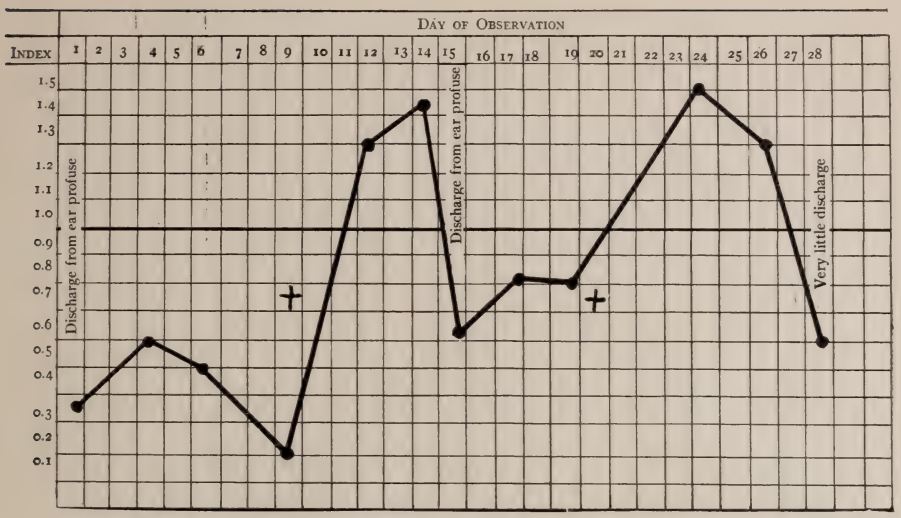

CHART 8.—The opsonic index in pseudodiphtheric (Group 1) otitis injected with autogenous vaccine. + = injection of vaccine.

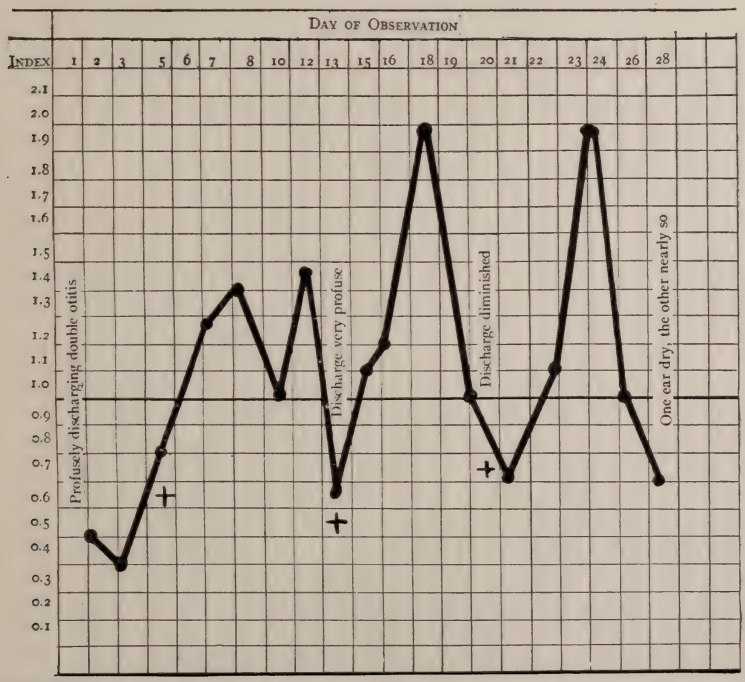

CHART 9.—The opsonic index in pseudodiphtheric (Group 2) otitis injected with autogenous vaccine. + = injection of vaccine.

It would, of course, be unwarranted to claim that the value of vaccine treatment is proved by events in these cases because, as I have said, acute suppurative otitis media so often heals rapidly under no particu-

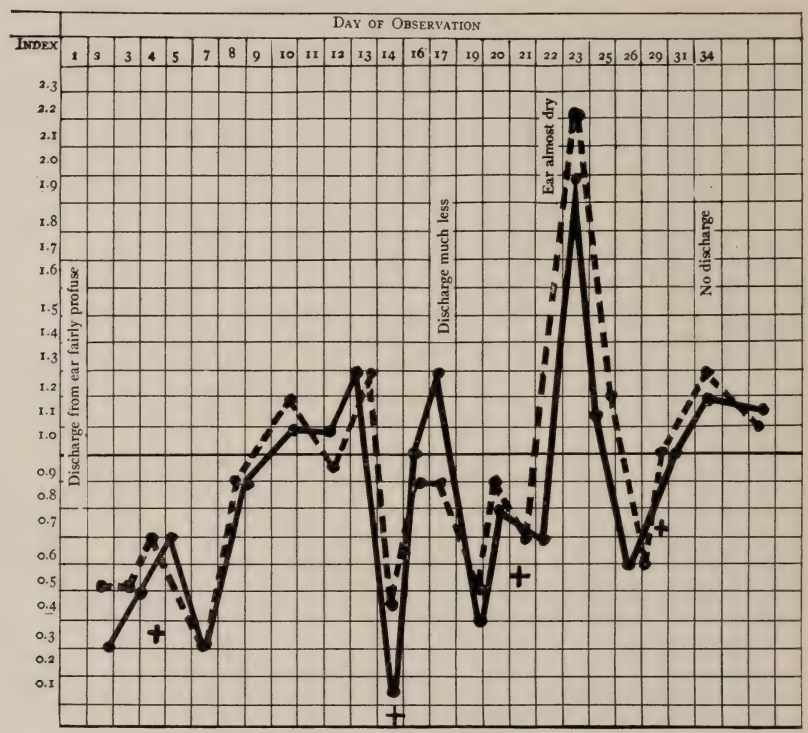

CHART 10.—The opsonic index in pseudodiphtheric (Group 1) otitis injected with autogenous vaccine.

NOTE.—The solid line represents the opsonic index as usually estimated—relation of the average number of bacteria taken up under the influence of the patient's serum per leucocyte to the average number of bacteria taken up per leucocytes in the pooled, normal serum—and the dotted line the proportion of phagocyting leucocytes in the patient's serum as compared with those in the pool. These two indices were estimated in the other cases also, the second serving as a control for the first. Wide variations seem to depend largely on an irregular distribution of the bacilli and with care the two indices usually come out very much alike. In all these experiments equal parts were used of washed blood, bacterial suspension, and serum.

lar treatment. But it seems reasonable to believe that patients who have a low opsonic index to the organism found in the pus from the ear will be benefited by a treatment which results in the raising of their index. The impression obtained so generally of the effects of vaccine therapy of staphylococcal infections warrants us in supposing

this to be true. It seems probable, then, that chronic suppurative otitis media may prove a fruitful field for this form of treatment.

SUMMARY.

Cases of otitis media which have pseudodiphtheria bacilli as the predominating organism in the pus from the ear have usually a low opsonic index for this bacillus.

In certain cases in which repeated examinations are made the index is found to cover a wide range, and the changes in the index often correspond to changes in the clinical symptoms.

By injections of dead cultures of the homologous strain the patient's opsonin to that strain can be increased. No ill effect follows such injections and an apparent improvement has resulted in several cases. The opsonin in the blood of these patients is specific for the variety of pseudodiphtheria bacilli to which the patient's strain belongs. This constitutes a strong proof that the pseudodiphtheria bacillus plays an etiologic rôle in such cases.

PSEUDODIPHTHERIA BACILLI AS THE CAUSE OF SUPPURATIVE OTITIS, ESPECIALLY THE POSTSCARLATINAL.*

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THE pseudodiphtheria bacillus has been a subject of dispute, of contradictory opinions, since it was first described; but upon one point there has been almost complete harmony. Whatever disagreement there may have been as to its morphology, its cultural and chemical properties, or its relation to the diphtheria bacillus, there has been a striking unanimity as to its unimportance.[1] Although it is often found in inflammatory processes, either alone or together with other organisms, still it is usually regarded as a harmless saprophyte, an accidental accompaniment of the pyogenic organisms which are really doing the work. Another view, not essentially different, is that this bacillus is only a non-virulent variety of the Klebs-Löffler bacillus.

The reasons for relegating the pseudodiphtheria bacillus to the rank of non-pathogenic organisms are apparently, first, that it is usually present on the normal nasal mucous membrane and very often on the normal conjunctiva, throat, external auditory canal, and urethra; second, that it is not virulent for guinea-pigs; and third, that when it is found in inflammatory exudates it is accompanied by various pyogenic bacteria.[2]

The literature of otitis media shows clearly the influence of this theory. The bacteriology of this disease has been thoroughly studied and the pseudodiphtheria bacillus is frequently mentioned as occurring in the pus from such cases; but almost invariably it is treated as unimportant and of no causative significance. Many otologists do

* Received for publication April 6, 1907.

[1] A full bibliography of this subject is given by Schabad, *Jarhb. f. Kinderheilk.*, 1901, 54, p. 381; Lewandowsky, *Centralbl. f. Bakt.*, 1904, 36, p. 339; Graham-Smith, *Jour. Hyg.*, 1904, 4, p. 258.

[2] There are a few exceptions to the majority. Thus Bergey attributes pathogenic properties to the pseudodiphtheria bacillus, which he found in 43 per cent of 30 specimens of pus examined (*Univ. of Penn. Med. Bull.*, September, 1905, p. 198). Kruse and Pasquale, Goldcheider, Leber, Robertson, McRae and Jeffery, Johnston and Goodall, and Howard, all believe that it is concerned in certain inflammatory processes.

not even mention it.[1] Yet when one examines the reasons for the belief that the pseudodiphtheria bacillus is always a harmless saprophyte they seem open to question. The wide distribution of these bacilli, their occurrence in normal ears, throats, noses, etc., is not an argument against their pathogenic character, for it would apply equally well to the streptococcus, the pneumococcus, and the staphylococcus albus. As to virulence for guinea-pigs, there have been many strains reported which were more or less virulent.[2] Nor can it be shown that the pseudodiphtheria bacillus when present in the pus of otitis media has no part in the inflammatory process. On the contrary there is evidence to show that it is in some cases the real cause of the disease. Thus Warnecke[3] found a non-virulent diphtheria-like bacillus in three cases of otitis media, once in pure culture. The first case was complicated with a progressive phlegmonous inflammation, the second with inflammation of the wound after an antrum operation, the third with meningitis and metastatic abscesses, and in all three cases the secondary lesions also contained the "xerosis" bacillus. L. D. Davis[4] isolated from 12 cases of scarlatinal otitis media a diphtheria-like bacillus, virulent but non-toxic, which she regarded as the cause of the suppuration. Egerton Williams[5] found diphtheria-like bacilli in 8 out of 62 cases of scarlatinal otitis media, and Duncan Forges[6] found them in 32 out of 40 cultures from similar cases. Both think the organisms attenuated varieties of diphtheria bacilli, but Graham-Smith, on the ground of his examination of the ear discharges of 10 scarlet fever patients, concludes that these non-purulent organisms described by Forbes and Williams were not diphtheria bacilli but only related forms, diphtheria-like.

As a result of the study of various forms of suppuration, especially suppurative otitis media, I have been led to the belief that certain varieties of pseudodiphtheria bacilli play a much more important part in such processes than is generally credited to them. The

[1] A good summary of the literature of this subject is found in Süpfle's articles, *Centralbl. f. Bakt.*, Abtheil I, 1906, Orig., 42, p. 304.

[2] See discussion of virulence in *Jour. Infect. Dis.*, 1904, 1, p. 690.

[3] *Münch. med. Wchnschr.*, 1900, 47, p. 1412.

[4] *Proc. New York Path. Soc.*, 1898, p. 170.

[5] *Brit. Med. Jour.*, 1901, 2, p. 1799.

[6] *Jour. of Path. and Bact.*, 1903, 8, p. 448.

material studied consisted of 52 cases of suppuration in various parts of the body, and 142 cases of purulent otitis media. The miscellaneous suppurations were almost equally divided between chronic and acute cases, and included abscesses, infected wounds, ulcers, osteomyelitis, three cases of purulent conjunctivitis, and three of pyorrhea alveolaris. The pseudodiphtheria bacillus was found in 11 cases, 21 per cent of all. In four of them it was in pure culture. The 142 cases of otitis media yielded a still larger proportion, for 35 per cent of these cases showed pseudodiphtheria bacilli as the predominating organism. The difference between these two groups of cases disappeared, however, when the post-scarlatinal ear cases were tabulated separately, for then it was shown that the large majority of pseudodiphtheria cases came under this head.

The following table gives the percentage of cases in which this bacillus was either the only or the most important bacterium found:

TABLE 1.

	No. of Cases	No. with Pseudodiphtheria Bacilli	Percentage with Pseudodiphtheria Bacilli
Acute scarlatinal otitis media	43	31	72%
Chronic scarlatinal otitis media	9	5	55
Acute non-scarlatinal otitis media	19	4*	21
Chronic non-scarlatinal ctitis media	71	11	15
Miscellaneous suppurations	52	11	21

* 1 meningitis, 1 influenza, 2 measles.

It seems impossible that the difference between the acute scarlatinal and the acute non-scarlatinal should be accidental. The number of pseudodiphtheria cases in chronic otitis is not so great as in acute, but the same proportion obtains between scarlatinal and non-scarlatinal chronic otitis as between scarlatinal and non-scarlatinal acute otitis. The pseudodiphtheria cases are more than three times as numerous in the post-scarlatinal class as in the non-scarlatinal. All the cases included in this list as yielding pseudodiphtheria bacilli contained these bacilli as the predominating organism. Those in which only a few were found were ruled out as doubtful. In 9 of the acute scarlatinal cases this bacillus was found in pure culture, in 16 more there were only a few colonies of other organisms. Two of the chronic cases were complicated with mastoiditis and the pseudodiphtheria bacillus was recovered in pure culture from the mastoid

wound as well as from the ears. In another case there was a profuse conjunctivitis and the pseudodiphtheria bacillus was found in the discharge from the eye as well as in that from the ear. If the pseudodiphtheria bacillus has no pathogenic character, if it is simply a normal inhabitant of the auditory canal, there is no way of explaining why it should be found so frequently in scarlatinal otitis media. In nine of the 43 cases it was found in pure culture, that is, in over 20 per cent.

The belief that these bacilli are actively concerned in otitis media, especially in scarlet fever, was strengthened by the study of two small ward epidemics in the department for contagious diseases of the Cook County Hospital. In one of them, all four children who developed this complication and in the other all five children, showed pseudodiphtheria bacilli in large numbers in the pus from the ear. The ward with the five cases was small and crowded and contained at different times eight children, five of whom had suppurative middle-ear disease. The first one, a profuse double suppurative otorrhea with pseudodiphtheria bacillus in the pus, was followed after four days by two others, in both of which this bacillus was the predominating organism. While these three children were still in this ward two fresh cases of scarlet fever were brought in, both of whom, after seven and eight days, developed suppurative otitis media with pseudodiphtheria bacilli, one case yielding a pure culture. The other organisms found in four of these cases were, twice the staphylococcus albus, once the streptococcus, and once, both of these. Not only were these last present in small numbers, but no one organism was found in all of the five cases except the pseudodiphtheria bacillus.

The 51 strains isolated from the cases of otitis media and six of those isolated from suppurative lesions elsewhere (the others were not studied in detail) fall into two clearly defined groups which correspond to the types described, more or less fully, by many observers, as pseudodiphtheria and xerosis bacilli or, more recently, as pseudodiphtheria and diphtheria-like or diphtheroid. They are quite distinct from the virulent bacillus, usually called the "Ruediger bacillus,"[1] being distinguished from it not only by fermentation tests,

[1] *Jour. Infect. Dis.*, 1904, 1, p. 690.

but by the action of specific bacteriolytic, agglutinative, and opsonic sera.

Group 1, to which 40 strains belong, consists of short rods, rarely granular or barred, which may or may not decolorize by Gram, growing abundantly on agar, forming a cloudy growth in broth, in short corresponding to the pseudodiphtheria bacillus as usually described. The typical Hofmann bacillus however is usually said to form no acid or a very slight amount of acid in sugar broth and to be devoid of virulence for guinea-pigs.[1] With Hiss' serum-water medium all of the strains of this group which were tested—35 in all—fermented dextrose and saccharose, and failed to ferment maltose, lactose and dextrin. As to virulence for guinea-pigs, the majority were not virulent, but three out of 12 strains when injected intraperitoneally produced a general invasion of the body and death in 24–48 hours. Diphtheria antitoxin did not protect against a lethal dose.

Group 2 resembles the Klebs-Löffler bacillus very closely. These are long-barred or granular bacilli, often clubbed, staining by Gram, growing rather scantily on agar, more abundantly on blood serum, and fermenting in Hiss' serum-water medium, dextrose, and maltose always, dextrin in 60 per cent of the strains tested, and lactose in 10 per cent. Saccharose was never fermented by this group. They are more often virulent than Group 1. Four of the seven strains tested killed guinea-pigs, but one of these strains, otherwise indistinguishable from the others, was by this test shown to be *B. diphtheriae* for diphtheria antitoxin served to protect against it. It is evident that there is no hard and fast line between this group and the true Klebs-Löffler bacillus, and when the organisms are non-virulent it is absolutely impossible to separate the two.

Eleven cases of ear suppuration yielded bacilli of this type, six alone, five in conjunction with a bacillus of Type 1. Although the two types found in my cases are quite distinct and remain so after long cultivation, they are apparently equally pathogenic for human beings and capable of producing the same clinical symptoms.

It is often suggested that the human tissue upon which the organism finds lodgment may modify the character of the latter, and that

[1] See Graham Smith, *Jour. Hyg.*, 1906, 6, p. 286; Knapp, *Jour. Med. Res.*, 1904, 12, p. 475.

the same organism may appear as short and solid rods in one throat or ear and as long and diphtheria-like rods in another. In the ward epidemic described above, the first case had only diphtheria-like bacilli, the second, third, and fourth had the short, solid variety, and the fifth had both; yet in these cases the infection apparently was carried from the first case to the others. A. P. Ohlmacher[1] has stated that a bacillus of the pseudodiphtheria type may change to a diphtheria-like bacillus when passed through the body of the susceptible guinea-pig, and that, conversely, a long, slender, barred bacillus may change to the short, solid form after passage through an insusceptible animal, such as the white mouse. In this way Spirig[2] explained the different varieties of *B. diphtheriae* found in different throats in a house epidemic where the source of infection for all cases was the same.

That the two varieties found in my cases of otitis media are distinct, though closely related, is shown by their response to the action of specific immune sera. The serum of a rabbit immunized against one type is bacteriolytic for that type and not for the other. Rabbits were immunized by repeated injections of a typical member of Group 1 and the serum tested on 14 members of this group and 6 of Group 2. At the same time the serum of rabbits immunized against the "Ruediger bacillus" was tested on the same organism. The serum of the rabbits immunized against a strain of Group 1 was bacteriolytic for and agglutinated 13 other strains of this same group, but the serum of the rabbits immunized against the "Ruediger bacillus" had no more effect upon them than normal rabbit serum. On the other hand the serum bacteriolytic for Group 1 had no effect upon the "Ruediger bacillus." Neither serum had any effect upon six strains of Group 2; indeed the bacilli grew more abundantly in the tubes with serum than in the controls. It has not been possible as yet to procure a serum bacteriolytic for Group 2, because rabbits have apparently little resistance to these bacilli and invariably emaciate and die. These tests serve to separate three varieties of the large, loosely defined family of bacteria known as the pseudodiphtheria bacillus into distinct though closely related groups.

[1] *Jour. Med. Res.*, 1906, 7, p. 128.

[2] Spirig, *Ztschr. f. Hyg.*, 1899, 30, p. 511.

SUMMARY.

Two varieties of pseudodiphtheria bacilli are found frequently in suppurative processes, especially in the pus of post-scarlatinal otitis media. In this disease they are found so frequently as to render it probable that they play an important part in its causation. No less than 72 per cent of 43 cases of acute scarlatinal otitis media gave cultures of these bacilli and 20 per cent gave pure culture. Only 21 per cent of the cases of acute non-scarlatinal otitis gave the same bacilli.

These bacilli fall into two groups. Group 1 ferments saccharose but not maltose, is seldom virulent for guinea-pigs, and is agglutinated and killed by the serum of rabbits immunized against one member of the group. Group 2 ferments maltose but not saccharose, is more often virulent for guinea-pigs, and is not affected by the serum of rabbits immunized against Group 1. Neither group is affected by an immune serum which is bacteriolytic and agglutinative for the "Ruediger bacillus."

The belief that these bacilli may cause suppurative otitis is greatly strengthened by the fact, as I show elsewhere,[1] that the opsonic index of the patients for these bacilli has been found to undergo marked variations, and by the further observation that the injection of corresponding vaccines appears to modify definitely the course of the infection.

[1] P. 313.

PHAGOCYTOSIS AND OPSONINS IN THE LOWER ANIMALS.*

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(*From the Marine Biological Laboratory, Woods Hole, Mass., Summer*, 1906.)

The discovery of opsonin by Wright and Douglas has been a great stimulus to the study of the finer mechanism of phagocytosis. This phenomenon is now receiving much attention, so far as concerns the leucocytes and serum of many warm-blooded animals, but nothing seems to have been done in the case of cold-blooded animals. During the past summer we made a study of the mechanism of phagocytosis by the leucocytes of a number of species of the lower animals, and the results of this study are given in the following tables.

In nearly all instances the blood was taken directly from the heart by means of a pipette and mixed immediately with half its volume of one per cent sodium-citrate solution. In the case of blood with serum of high osmotic pressure, enough sodium chloride was added to the sodium-citrate solution to bring the osmotic pressure of the solution approximately up to that of the serum. Whenever the corpuscles were washed sodium-chloride solution having an osmotic pressure approximately equal to that of the serum was used. Frequently it was found necessary to add a trace of sodium citrate to the sodium-chloride solution to prevent clotting of the corpuscles. The mixtures of corpuscles and bacterial suspension were kept at room temperature (summer) for one hour, then smears were made and stained, and the average number of bacteria ingested by each leucocyte determined by counts in 20 or more leucocytes. Sensitization of bacteria was affected by suspending them in the serum for 30 minutes, centrifugating to remove the serum, and washing the bacteria once or twice in a large quantity of NaCl solution. In the sensitization experiments great difficulties were sometimes encountered by the firm agglutination of the bacteria.

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Table 1 shows that the leucocytes in the blood of nearly all species used ingest the ordinary pathogenic bacteria in large numbers. Not

TABLE 1.

PHAGOCYTOSIS OF BACTERIA BY LEUCOCYTES IN BLOOD OF VARIOUS COLD-BLOODED ANIMALS.

	Staphylo-coccus	Strepto-coccus	Pneumo-coccus	Coli communis
Snapping turtle (Chelydra serpentina)	20+	20+	20	20+
Painted turtle (Chrysemys picta)	11	14	1	15
Bullfrog (Rana)	40	13	..	30
Squeteague (Labrus squeteague)	..	9.9	..	11
Flounder (Pseudopleuronectus Americanus)	..	7.5	..	4
Conger eel (Conger oceanicus)	6.6	11	1.6	16
Dogfish (Mustelus canis)	25	4	..	6
Sand Shark	20	12	..	+
Skate (Raja erinacea)	0	0	..	..
King crab (Limulus)	..	8.7	..	..
Clam (Venus)	30	31	..	..

all leucocytes in the smears showed phagocytosis and in the counts those not engaged in phagocytosis were usually disregarded. This was found necessary because there are some leucocytes in these animals which appear never to act as phagocytes and it is extremely difficult to distinguish these from phagocytic cells. In three experiments with the blood of the summer skate no phagocytosis was obtained.

Table 2 shows that there is no phagocytosis of untreated staphylococci by the washed leucocytes of these animals, but there is phagocy-

TABLE 2.

THE IMPORTANCE OF SERUM IN PHAGOCYTOSIS OF BACTERIA BY THE LEUCOCYTES OF COLD-BLOODED ANIMALS.

				Phagocytosis
Washed dogfish corp.	+	Staphylococcus		0
" " "	+	"	+dogfish serum	25
" " "	+	"	sensitized in dogfish serum	20
Washed sand-shark corp.	+	"		0
" " "	+	"	sensitized in shark serum	20
Washed snapping-turtle corp.	+	"		1
" " "	+	"	+turtle serum	20
Washed frog corp.	+	"		2.7
" " "	+	"	+frog serum	40

tosis in these mixtures if normal serum is added to them. Furthermore, there is good phagocytosis by the washed leucocytes if the cocci are sensitized in the serum before they are added to the suspension of leucocytes; that is, the action of the serum on the cocci is essential before phagocytosis can take place. In the sensitization it is not necessary that the serum used comes from an animal of the same

species as that furnishing the leucocytes or from a closely related species. Table 3 shows that sensitization is very successful also with

TABLE 3.

PHAGOCYTOSIS OF BACTERIA SENSITIZED BY HETEROLOGOUS SERA.

						Phagocytosis
Washed human corp.	+	Staphylococcus	sensitized in	human serum		17.
" " "	+	"	"	" Limulus serum		7.7
" " "	+	"	"	" sand-shark serum		9.0
" " "	+	"	"	" snapping-turtle serum		15.0
" " "	+	"	"	" Phascalosoma serum		9.9
" " "	+	"	"	" Holothurian serum		14.5
" " "	+	"	"	" Arbacia serum		20.0
" " "	+	"	(not sensitized)			1.8
Washed dogfish corp.	+	"	sensitized in	dogfish serum		25.0
" " "	+	"	"	" sand-shark serum		17.8
" " "	+	"	"	" painted-turtle serum		20.0
" " "	+	"	"	" Limulus serum		20.0
" " "	+	"	"	" human serum		10.0
" " "	+	"	(not sensitized)			0.0
Washed sand-shark corp.	+	"	sensitized in	sand-shark serum		20.0
" " "	+	"	"	" dogfish serum		20.0
" " "	+	"	"	" painted-turtle serum		20.0
" " "	+	"	(not sensitized)			0.0

heterologous sera. Indeed, the serum of some of the very lowest forms of animal life, such as the sea-urchin, may be used to sensitize staphylococci for phagocytosis by human leucocytes. Variable results are frequently obtained in these sensitization experiments, which may be due to the fact that some of these sera may agglutinate the cocci very firmly, making phagocytosis practically impossible. Some sera, namely, that of the summer skate, squeteague, flounder, sea-robin, lobster, and spider crab, entirely failed to sensitize the cocci for phagocytosis by human leucocytes. Our experiments have shown that many sera of cold-blooded animals are highly toxic for human leucocytes. If the leucocytes are left for one hour in the serum of conger eel, dogfish, flounder, squeteague, skate, or frog, the nuclei are vacuolated and usually considerably disintegrated. The chromatin is often found in fine shreds scattered throughout the cytoplasm of the cell. In these experiments the sera were diluted with twice their volume of normal salt solution or salt solution and distilled water. The distilled water was used when the serum normally had a much higher osmotic pressure than that of human serum. Heating these sera to 55° C. for 30 minutes destroys the leucotoxin.

Table 4 shows that the opsonin in the sera of cold-blooded animals is a thermolabile substance, being destroyed by heating at 55° C. for

TABLE 4.

THE EFFECT OF HEAT ON OPSONINS OF COLD-BLOODED ANIMALS.

		Phagocytosis.
Washed dogfish corp.	+Staphylococcus	0
" " "	+Colon bacillus	0
" " "	+Staphylococcus+dogfish serum	25
" " "	+ " + " " heated 55° 30′	0.2
" " "	+Colon bacillus+ " "	20
" " "	+ " + " " heated 55° 30′	0.5
" " "	+Staphylococcus+Limulus serum	20
" " "	+ " + " " heated 55° 30′	0
Washed snapping-turtle corp.	+ "	1
" " "	+ " +Turtle serum	20
" " "	+ " + " " heated 55° 30′	2.9
Washed land-shark corp.	+ " +shark serum	14.0
" " "	+ " + " " heated 60° 30′	0
Washed human corp.	+ " +Limulus serum	6.9
" " "	+ " + " " heated 50° 15′	7.0
" " "	+ " + " " heated 55° 15′	0.8

30 minutes. Not many experiments were made to determine the exact temperature at which these opsonins are destroyed but in the case of the Limulus it was not destroyed at 50° C. in 15 minutes.

CONCLUSIONS.

1. The unwashed leucocytes of many forms of cold-blooded animals readily take up *in vitro* the common forms of bacteria (staphylococci, streptococci, pneumococci, colon bacilli).

2. Washed leucocytes of many of the lower forms do not take up bacteria; but if the latter are sensitized in the homologous or in many heterologous sera they readily undergo phagocytosis by the washed leucocytes.

3. Bacteria sensitized in sera of cold-blooded animals may be readily taken up by the washed corpuscles of warm-blooded animals; and vice versa.

4. Phagocytosis is prevented or greatly inhibited by heating the various sera to 55° C. for 30 minutes.

5. In some cases phagocytosis was not obtained by adding sera of certain animals to heterologous corpuscles. In such mixtures there is evidence that substances are present in the serum which are toxic for the leucocytes and thus may prevent phagocytosis.

6. Phagocytosis in representative forms of all the great groups of animals down to and including the echinoderms (Echinodermata, Mollusca, Vermes, Arthropoda, Vertebrata) seems to be largely dependent on the presence of opsonins in the sera.

A CONTRIBUTION TO THE STATISTICS ON THE PRESENCE OF DIPHTHERIA BACILLI IN APPARENTLY NORMAL THROATS.*

REPORT OF 1,000 CASES EXAMINED (1905–6).

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ALMOST since the discovery of the diphtheria or Klebs-Löffler bacillus, the presence of these organisms or morphologically similar organisms in the throats and noses of persons showing no clinical evidence of diphtheria has been recognized and numerous investigations to determine their mode of entrance, their frequency, their meaning, and virulence, etc., have been made.

According to R. O. Neumann, virulent diphtheria bacilli are **never** found in **normal** throats and noses, but in those cases where catarrhal conditions are present. Of some 206 people examined by him, of whom 111 were normal and 95 were suffering from nasal troubles, none with normal mucous membranes harbored virulent diphtheria bacilli, whereas 8 per cent of those suffering from acute and chronic catarrhs showed true virulent diphtheria bacilli. Pseudodiphtheria bacilli, under which he includes both *B. xerosis* and Hoffman's bacillus, were isolated from 98 per cent of the normal and 97 per cent of the affected cases, and are considered by him as normal inhabitants. Other observers, chiefly the earlier ones, give on the other hand the impression that the Klebs-Löffler bacillus is fairly common in the mouths of normal persons, and this impression prevails to large extent among physicians, who are inclined to think the condition so common that it can be ignored. Still others show that there is a certain small percentage of cases which harbor diphtheria bacilli where, as far as can be discovered, no recent and direct exposure to or previous infection by diphtheria can be traced. These cases are found, however, rather in cities and in institutions where diphtheria is always present to greater or less extent.

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Steenmeyer investigated a small series of children from a village where no diphtheria had existed for 10 years and compared these with a similar number of children taken from the surgical wards of a hospital in Rotterdam. Of the former, 52.4 per cent showed organisms resembling diphtheria bacilli (pseudodiphtheria probably included?) but none was virulent, whereas, the city children gave a percentage of 75.6 similar organisms and of these 7.3 per cent were virulent. Of the 330 normal non-exposed persons examined by Park and Beebe (1894) in N. Y. City, 9.7 per cent showed organisms morphologically similar to the diphtheria bacilli, but only 2.4 per cent were virulent[1] to animals. One-fourth of those infected with virulent bacilli subsequently came down with diphtheria. Fibiger in Copenhagen (1895) found 11.1 per cent non-virulent diphtheria-like organisms in healthy mouths and 2.9 per cent virulent Klebs-Löffler bacilli. Kober (1899) in Breslau examined 600 school children and found 1.6 per cent harbored non-virulent and 0.83 per cent virulent diphtheria bacilli. In all of these infected children he traced exposure to diphtheria, more or less remote. Denney (1900) out of 235 normal persons, and Graham-Smith, out of 362, found none infected with virulent Klebs-Löffler bacilli; those found were non-virulent. E. Miller (1896) examined a series of 100 children on admission to an institution and found 24 per cent with diphtheria-like bacilli in their throats. Of the 12 tested on animals, 6 were virulent. In only one case of the 24 was there known exposure to diphtheria. Later 18 children in the same institution; who had negative cultures on admission, gave positive cultures after being in the house for some time. Four of these were virulent. There are many other investigators who have found varying percentages of infected throats, Stross, Cobbett, Gross (7.9 per cent), Hewlett and Murray (15 per cent), etc., but on the whole their statistics are not very valuable for our purpose, chiefly because of the incompleteness of their virulence tests.

Recently, January 1907, M. E. Pennington of the Philadelphia Health Bureau has published the results of her investigations among well school children. From 25 schools taken at random, she found 9.3 per cent of the children infected with diphtheria-like organisms. About one-half of these organisms were to a greater or less degree pathogenic to guinea-pigs and 13.5 per cent were highly virulent. Some of these children were exposed to diphtheria.

Unless the conditions are parallel in the different investigations, or unless these are taken into account, it is manifestly unfair to compare statistical results. That virulent diphtheria bacilli are found in normal throats of persons exposed to diphtheria is unquestioned, but how far they are present in normal non-exposed persons is still a mooted point. The results depend largely on what each individual investigator considers as a "normal throat," how far he differentiates the members of the group of organisms showing the morphological characteristics of the diphtheria bacillus, on the environment of the cases examined, and the severity of the disease as it exists in the community. In a city like New York, where there are some 8,000–12,000 cases of diphtheria reported each year and the possible

[1] By virulent we mean producing diphtheria toxin.

sources of infection are so many, tenements, schools, shops, public conveyances, hotels, restaurants, etc., it is almost impossible to trace in every case exposure to the infection. It is certainly most plausible to believe that infection always comes from a previously infected individual, whether it be mediate or direct, remote or recent.

With this in mind the object of the present investigation was not to prove or deny the presence or absence of true diphtheria bacilli in the healthy throats of persons *never* exposed to diphtheria, but simply to determine approximately in what number of the apparently normal throats of individuals among whom diphtheria is most frequent, viz., children in crowded tenements and institutions, virulent diphtheria bacilli are found. Two series of cases were examined, first, those as far as known not recently or directly exposed to diphtheria; second, those directly and definitely exposed in their families.

SERIES I.

The first series of cases examined (1,000 in all) were, with a few exceptions, taken from among the tenement-house children who came for treatment to the dispensaries and hospitals of the city and from the nurseries of the N. Y. Foundling Asylum. As far as possible inquiry into immediate and recent exposure to diphtheria was made and noted, and no child suffering from an angina or anything suggesting a "sore throat," nasal discharge, or laryngitis, was included in the series. There was no routine examination for adenoids, large tonsils, or abnormalities of the nose, but a clean fauces alone was considered "normal."

Of the 18 cases in which virulent Klebs-Löffler bacilli were found there was only one in which a possible history of exposure several weeks before was obtained. In the children from the hospital wards and nurseries, while there was no definite exposure to clinical diphtheria, so-called "laboratory cases" with bloody nasal discharge had been in the wards for short intervals until the clinical symptoms developed. In the Babies' Hospital there were four such nasal cases in the wards for one or two days during March and April, from which virulent diphtheria bacilli were isolated. One of these children also had non-virulent diphtheria bacilli in his throat. This child was considered to have given diphtheria to the nurse who took

care of him (he was isolated as soon as the bacilli were discovered) and to another child. The one "normal" child in the Babies' Hospital from whom virulent organisms were isolated was down on the records as having been exposed to diphtheria and had been given an immunizing dose of antitoxin during a previous admission three months and a half before. He also subsequently developed a bloody nasal discharge from which Klebs-Löffler bacilli were isolated.

In another hospital from which no positive cultures were obtained from any of the normal children, there was in the same ward a child suffering from a post-diphtheric paralysis, from whose throat virulent Klebs-Löffler bacilli were obtained.

With the exception of 50 children, cultures were taken from the throats alone, so that it is very possible the percentage of bacilli found might have been somewhat higher, especially of the pseudo-diphtheria bacilli, if nasal cultures had been made at the same time. Wesbrook, Wilson, and McDaniel (1899) found this true among the children in a boarding-school investigated by them. To determine how far this might be so in our cases, 50 children were examined, cultures being taken from each side of the nose and from the throat (Table 1). In two per cent of the cases virulent Klebs-Löffler bacilli were found in the nose alone. Pseudodiphtheria bacilli were found about twice as often in the nose as in the throat.

TABLE 1.

NOSES VS. THROATS.

Source	Number Examined	Ages	Diphtheria-like Bacilli Isolated from Nose Alone	Diphtheria-like Bacilli Isolated from Throat Alone	Diphtheria-like Bacilli Isolated from Both	Virulent	Non-virulent	*B. Xerosis* from Nose Alone	*B. Pseudodiphtheria* from Total Noses	*B. Pseudodiphtheria* from Total Throats	*B. Pseudodiphtheria* from Both Nose and Throats
Babies' Hospital wards.....	28	2 weeks to 3 yrs.	1	0	0	1	0	0	23	10	10
N. Y. Infirmary dispensary.....	22	2 mos. to 10 yrs.	0	0	1	0	1	1	9	5	3
Total.....	50		1 2%	0	1	1 2%	1	1	32 64%	15 30%	13 26%

The technique was that in general use. Löffler serum tubes were inoculated according to the usual board of health directions and examined the following day, after incubation. From all the tubes

that were positive or suspicious, plates and ascitic broth tubes were inoculated. After isolation of the organisms, with the exception of the pseudodiphtheria or Hoffman's bacillus, all were tested in the usual way on guinea-pigs for virulence. Guinea-pigs weighing from 200 to 300 grms. were inoculated subcutaneously at the outset with 1 c.c. and 0.5 c.c. respectively of 48-hour ascitic broth cultures of the strain tested, each pig being controlled by a similarly inoculated animal receiving in addition a counteracting dose of diphtheria antitoxin. The strains that killed pigs within the four days were again tested in every case with smaller doses (always controlled by antitoxin) up to 0.1 c.c. When the initial injection did not kill, larger doses sometimes up to 10 c.c were given. Table 2 shows the relative toxicity of the 56 strains isolated.

TABLE 2.

VIRULENCE TESTS.

(The doses cited are the minimum that killed and the maximum that did not kill.)

Death of Unprotected Pig Within	Number Receiving 0.1 c.c.	Dose 0.2 c.c.	Number Receiving 0.5 c.c.	1 c.c.	2 c.c.	5 c.c.	10 c.c.
24 hours	2			1*			
48 hours	3	1	6				
72 hours	1		1				
4 days			1	1			
3 weeks				1			
Non-virulent				26	3	1	8

*Virulence of this strain is doubtful as 0.5 c.c. from same broth culture did not kill when inoculated subcutaneously into 250 grm. guinea-pig; virulence test was not repeated.

The pigs charted above as killed by 1.c.c., 0.5 c.c., 0.2 c.c., all received smaller doses up to 0.1 c.c., which did not kill.

Where there was any doubt as to the identity of a supposed pseudodiphtheria bacilli, cultural tests and animal inoculations were used to establish it. The organisms were classified as follows: (1) true virulent Klebs-Löffler bacilli; (2) non-virulent diphtheria-like bacilli; (3) xerosis bacilli; (4) pseudodiphtheria or Hoffman bacilli (alkali producing in glucose broth).

From Table 3, it will be seen that in four instances organisms which behaved somewhat differently culturally from the ordinary diphtheria bacillus were isolated. The growth on Löffler serum was very delicate and the colonies sometimes were not visible for 48 hours. They did not produce toxin and were identified as *B. xerosis*. In

10 c.c. doses, in one case the pig controlled by antitoxin died, in another both pigs died, and in the remaining two neither animal died. From one child this organism and a non-virulent diphtheria-like organism were isolated at the same time.

Tables 3, 4, and 5 give the results of the investigations with the number of cases examined, according to season, ages, and source of material respectively.

TABLE 3.

NORMAL THROATS. SEASON.

1905–6	Number of Cases Examined	Virulent Klebs-Löffler Bacilli in	Non-Virulent Diphtheria-like Bacilli in	Total Diphtheria-like Bacilli in	Pseudodiphtheria Bacilli (Hoffman's)	*B. xerosis*
August 30–31, 1905	21	0	0	0	0	
September	30	0	1	1	1	
October	31	0	0	0	0	
November	10	0	0	0	6	
December	27	0	4	4	4	
January, 1906	69	2	3	5	35	2
February	56	1	2	3	20	1
March	107	3	3	5	24	
April	37	0	0	0	6	
May	152	3	6	9	18	
June	94	2	7	10	50	1
July	169	4	10	14	47	
August	190	3	2	5	52	
October	7	0	0	0	3	
Total	1,000	18 (1.8%)	38 (3.8%)	56 (5.6%)	266 (26.6%)	4 (.4%)

TABLE 4.

NORMAL THROATS. AGE.

Ages	Number Cases Examined	Total Diphtheria-like Bacilli Isolated from	Virulent Klebs-Löffler Bacilli in	Pseudodiphtheria Bacillus (Hoffman's b.) in
Under 1 year	183	6	3	50
1–2 years	141	7	6	45
2–3 years	112	6	4	33
3–4 years	131	11	4	35
4–5 years	107	8	2	29
5–6 years	61	4	2	25
6–12 years	184	12	1	46
12–20 years	33	0	0	3
20+ years	29	1	0	0
Unknown	19	1	0	0
Totals	1,000	56	18	266

A casual examination of Table 3 would lead one to infer that a larger number of diphtheria-like organisms were isolated in the summer months during the period when diphtheria is least prevalent. This is due not only to the fact that more cases were examined during the latter six months of the investigation, but chiefly because the majority of the children examined, in contrast to the earlier

TABLE 5.
NORMAL THROATS. SOURCE.

Source of Cases	Number of Cases	Diphtheria-like Bacilli found in	Virulent Klebs-Löffler Bacilli Found in
New York Infirmary for Women and Children dispensary	341	24 or 7 %	9 or 2.6 %
New York Babies' Hospital dispensary	216	7 " 3.2	1 " .46
New York Babies' Hospital wards	125	4 " 3.2	1 " .8
New York Foundling (nurseries)	115	14 " 12.1	4 " 3.5
St. Mary's Hospital, surgical and medical wards	44	0	0
Bellevue Hospital, surgical and medical wards	56	3 " 5.2	0
Contagious Disease Hospital, Kingston Ave., Brooklyn, scarlet-fever and measles wards	61	3 " 4.9	2 " 3.2
NewYork Polyclinic dispensary and wards	42	1 " 2.4	1 " 2.4
Total	1,000	56	18

cases, were those in the hospital wards and the nurseries. For example, all of the cases taken during July came from the New York Foundling, Bellevue, the Babies', and St. Mary's Hospitals, likewise the majority of those examined during June and August, whereas previous to March all came from the out-patient clinics.

The children from the Foundling Asylum were all healthy children from the nurseries, playing around in the open air on the roofs almost all day, but sleeping in the same dormitories at night. They averaged from 2 to 5 years of age.

The children at the Babies', St. Mary's, and Kingston Avenue Hospitals were confined to the wards all the time. Some of the surgical cases from Bellevue were out in the open air part of the time. From the Kingston Avenue Hospital only one child from each of the scarlet-fever and measles wards harbored virulent diphtheria bacilli.

In New York City the mortality from diphtheria in the last 13 years has gradually dropped from 36.4 per cent in 1893, to 10.3 per cent in 1905. Whatever this be due to, whether the earlier recognition and treatment of the disease, better isolation, the use of antitoxin, or the natural wave-like variation in the intensity of the disease, we might at the same time expect a corresponding diminution in the number of normal people infected. In comparing our results with those of Park and Beebe (1894) with whose investigation ours is more nearly analogous, we find the decrease not commensurate. However, while the mortality has decreased since then, the average yearly number of persons reported to the Health Department as clinic-

ally infected with diphtheria has remained practically the same, and because of the comparative mildness of the disease, the number of unrecognized anginal cases and those simulating tonsilitis probably contribute to the spreading of the infection.

SERIES 2.

The second series of cases examined were throat cultures from the immediate family of patients suffering from clinical diphtheria. In all, 202 cases were investigated, extending over a period of four months from April through July, 1906. The cultures were obtained through the courtesy of the medical inspectors of the Health Department, who took the cultures and forwarded them to us.

Park and Beebe's investigations of contact cases in 1894 showed 50 per cent of the throats infected with virulent Klebs-Löffler bacilli Of these 40 per cent subsequently developed diphtheria. These

TABLE 6.

CONTACT CASES. SEASON.

1906	Number Cases Examined	Total Diphtheria-like Bacilli in	Virulent Klebs-Löffler Bacilli in	Non-Virulent Diphtheria-like Bacilli	Pseudodiphtheria Bacilli (Hoffman's)	*B. xerosis*
April	148	13	11	2	19	
May	10	2	0	2	2	
June	2	0	0	0	2	
July	42	5	3	2	2	
Total	202	20	14	6	25	

TABLE 7.

CONTACT CASES. AGE.

Ages	Number Cases Examined	Total Diphtheria-like Bacilli Isolated from	Virulent Klebs-Löffler Bacilli in
Under 1 year	9	1	1
1–2 years	4	0	0
2–3 years	17	3	1
3–4 years	4	0	0
4–5 years	10	1	1
5–6 years	11	2	1
6–12 years	42	6	5
12–20 years	20	0	0
20+ years	65	6	5
Unknown	20	1	0
Total	202	20	14

persons all lived crowded together under the most unfavorable surroundings, often sleeping several in a bed with the infected patient. A second series of cultures from children living in more favorable conditions showed virulent bacilli in only 10 per cent. Kober found

virulent bacilli in 8.1 per cent of contacts, Hollstrom in 18.8 per cent, Aaser 20 per cent, Denny 11 per cent, Goadley 34.1 per cent (1900), Graham-Smith (1902) 10.4 per cent, etc. Thus the percentages vary according to conditions of environment, disinfection, etc.

From our 202 cases, organisms resembling diphtheria bacilli were isolated 20 times, 14 virulent and 6 non-virulent, that is, 7 per cent of the individuals were infected with virulent Klebs-Löffler bacilli. The subsequent history of these persons was not followed. The majority were older than those of the first series, more adults being included.

CONCLUSIONS.

1. Diphtheria-like organisms are present in a certain number of apparently normal throats, even where exposure to infection by diphtheria cannot be traced.

2. A certain proportion of these organisms (in our cases about one-third) are virulent, and the persons thus infected are a potential source of danger to the community while about two-thirds of the organisms are not true diphtheria bacilli and are probably harmless.

3. The other conditions being the same, virulent Klebs-Löffler bacilli are found in the mucous membranes of those exposed, about four times as often as in those apparently not exposed.

4. Mild sore throats and "colds" with bloody nasal discharge, such as are fairly common in children, should not be lightly considered. Whenever possible, cultures should be taken, and if diphtheria-like organisms are found, their virulence should be tested.

I wish to express my thanks to Dr. Park and Dr. Williams for their oversight of this work, and to Dr. Southworth, Dr. Annie Daniel, Dr. Ethel Brown, and the house officers of the various hospitals, for their courtesy in allowing me to obtain the material.

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TUBERCULOSIS OF THE PLACENTA.

A HISTOLOGICAL STUDY WITH ESPECIAL REFERENCE TO THE NATURE OF THE EARLIEST LESIONS PRODUCED BY THE TUBERCLE BACILLUS.*

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IN this *Journal* for January, 1904, the writer, with Dr. D. M. Cowie, contributed a study of placental and congenital tuberculosis based upon a case observed by them of placental tuberculosis occurring in the seventh month of pregnancy, associated with miliary tuberculosis of the mother. At the time only eight undoubted cases of tuberculosis of the human placenta could be collected from the literature. Of these, two cases had been reported by Lehmann, three by Schmorl and Kockel, two by Warthin, and one by Auché and Chambrelente. Since our paper was published a number of other cases have been recorded. Of these, one case by Runge had been reported while our article was in press; since its publication Schmorl and Geipel have added nine cases, and Wollstein one, the total number at the present thus being brought up to 20. This rapid increase in the number of reported cases bears out the belief expressed in our paper that placental tuberculosis is probably of much more frequent occurrence than is at the present time supposed, and that with more careful and systematic examinations of placentas the number of cases would increase. This view found striking corroboration in the examination by Schmorl and Geipel of placentas from 20 cases in which the mothers were tuberculous, and the demonstration of tuberculosis of the placenta in nine of these cases. The fact that the majority of reported cases were seen by but two or three observers also points to a not infrequent occurrence of the condition.

Aside from the question of its frequency and the part played by placental tuberculosis in the etiology of congenital tuberculosis, a number of problems were suggested by the histological study of our case that could not at the time be satisfactorily answered. The

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part played by the syncytium in the development of the placental lesion, the primary or secondary nature of the agglutination-thrombi in the intervillous spaces, the histogenesis of the giant-cells, the passage of tubercle bacilli through an apparently normal syncytium, etc., constituted some of the questions that it was hoped a more extended study of the material might serve to answer. The purpose of the present paper is to present the results of such a study.

Recalling briefly the clinical history of our case, the mother, aged 27, had a bad family history, her father, three aunts, and one brother having died of pulmonary tuberculosis. She herself had been a frail child. Previous to and after her marriage she had urinary difficulty. During the last year of her life she developed a dry and distressing cough with attacks of sweating. She nevertheless became pregnant, and while in the seventh month died of general miliary tuberculosis. The fetus and placenta were expelled twelve hours before death, no signs of life being shown in the child. Autopsy of the mother showed a chronic tuberculosis of the kidneys and an acute general miliary tuberculosis. The fetus, membranes, placenta, and the uterus with its appendages were secured entire for study. Animal inoculations from the fetal liver and the placenta gave positive results. Tubercle bacilli were found in numbers in the blood-vessels of the fetal liver, but no tubercles were seen in any of the fetal organs or tissues. Countless tubercles in all stages of development were found in the placenta, and numerous tubercle bacilli were demonstrated in these. Animal inoculations with placental blood also gave positive results.

The entire uterus, membranes, placenta, fetus and cord described above were preserved after the original examination, and from time to time, as circumstances would permit, a more minute and thorough study has been carried out than was at first possible. It is to the results of this study that I now wish to direct attention, particularly with reference to the changes produced in the decidua and placenta by tubercle bacilli, and at the same time to fix more precisely the character of the primary lesion of tuberculosis. The entire placenta was cut into parallel strips about 5 mm. wide. Half of these were fixed in mercuric chloride, half in 4 per cent formaldehyde, after-hardened in alcohol, and imbedded in paraffin. The uterus was cut into suitable blocks and similarly treated. During the last two years, with the assistance of advanced students, thousands of sections have been cut from this material and have been studied. A large portion of it has also been utilized as teaching material, and I have in this way been enabled to examine microscopically the greater part of the placenta and decidua of this case. From this this study I now feel that certain definite answers can be given to

some of the problems mentioned above. To the laborious study of a great number of consecutive serial sections are due the conclusions herein contained. The majority of the sections were stained with hematoxylin and eosin for the histological study, although a variety of special stains was employed for different purposes. For the demonstration of the tubercle bacilli the carbol-fuchsin method was used.

Schmorl and Geipel in their work on placental tuberculosis describe it as occurring in four forms, dependent upon the localization of the tuberculous lesion: (1) on the periphery of the villi; (2) in the stroma of the villi; (3) in the basal decidua; (4) in the chorion involving the amnion as well. From my own studies of placental tuberculosis I should class the lesions as follows: (1) decidual; (2) intervillous; (3) intravillous; (4) intravascular chorionic; (5) chorio-amniotic. This agrees with the classification given by Schmorl and Geipel with the addition of the intravascular chorionic tubercles, which are due to the development of tubercle bacilli in the blood-vessels of the villi or chorionic stems, resulting in a primary lesion of the endothelium of the vessel, followed by a secondary thrombosis. The later organization of the thrombus by epithelioid cells derived from the connective-tissue of the vessel wall gives rise to an intravascular tubercle. These five types of placental tuberculous lesions will now be described in detail.

A. TUBERCULOSIS OF THE DECIDUA.

Throughout the entire decidua, in both the superficial and basal portions, there were present areas of necrosis of varying size. Many of these areas were fairly sharply circumscribed, but the majority were surrounded by a narrow zone of necrobiotic decidual cells. From the areas of necrosis found normally in the decidua of the mature placenta these necrotic foci could as a rule be distinguished by the caseous character of the necrosis and the marked karyorrhexis of the lymphocytes and polymorphonuclear leukocytes found in and about the area. They took both eosin and hematoxylin more heavily than normal necrotic areas, appeared somewhat less translucent and contained more fibrin, as was shown by staining with Weigert's fibrin method. Disintegration and diffusion of the chromatin from the decidual cells and leukocytes gave to the smaller areas, as well as to the peripheral portions of the larger ones, a diffuse blue staining with hematoxylin. The centers of many of the larger foci showed a partial liquefaction, small pockets of soft caseous matter thus being formed. This change was noted particularly in those foci containing a large number of polymorphonuclear leukocytes. The decidual lesions presented all stages of the necrotic process, from small groups of decidual cells showing beginning disintegration up to the larger areas of complete necrosis.

Associated with the necrotic areas in the decidua there were always present thrombi in the decidual sinuses or vessels, the necrotic areas usually lying adjacent to or surrounding a thrombosed vessel. The smallest of the thrombi were composed of a deep pink-staining granular or hyaline substance derived from agglutinated red-blood cells or blood-plates. Superimposed upon the hyaline thrombi laminated masses of fibrin were found in the larger vessels and sinuses. These larger thrombi often extended out into the intervillous spaces. Serial sections showed that in all

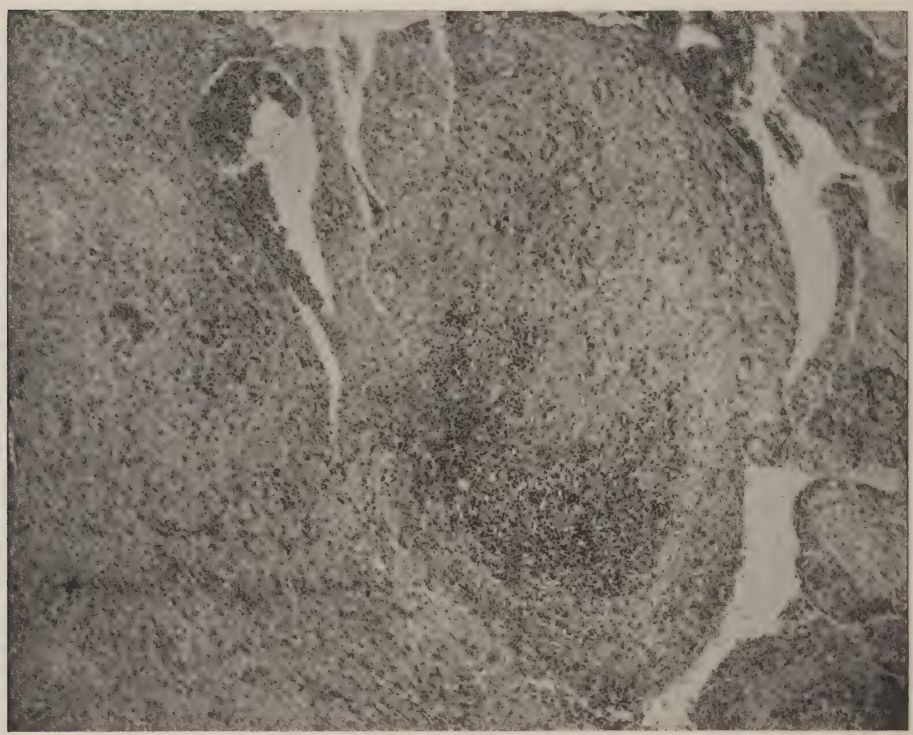

FIG. 1.—Early stage of tuberculosis of decidua. Area of beginning caseation.

cases the hyaline agglutination-thrombus rested upon or was adherent to a portion of the wall of the blood-vessel or sinus, that showed varying stages of necrobiosis or necrosis at the point of attachment. In the case of the smallest thrombi the degeneration or necrosis involved only the endothelium or syncytium of the vessel or sinus and the decidual cells lying just adjacent to the vessel wall. The varying stages of the process found and the relations between the thrombus and the lesion of the vessel wall led me to conclude that in all cases the thrombus is a secondary formation dependent upon a local injury to the vessel lining. As a result of this local lesion there is deposited upon the damaged area a mass of blood-plates and red-blood cells. These agglutinate to form a hyaline thrombus. In and about this lymphocytes and polymorphonuclear

leukocytes collect and quickly undergo karyorrhexis. Following these events there is a secondary coagulation with the formation of thready fibrin. With the growth of bacilli in the thrombus it undergoes caseation necrosis and this process gradually involves the neighboring decidua.

The staining of sections of the decidua with carbol-fuchsin, decolorization in nitric acid, and counterstaining in methylene-blue, showed the presence in the necrotic foci and in the hyaline thrombi of tubercle bacilli. In the very small hyaline thrombi

FIG. 2.—Typical area of tuberculous necrosis and thrombosis in decidua.

associated with slight degenerative changes in the wall of the vessel the bacilli were found only in the thrombus; but in the larger areas of necrosis of the decidual cells the bacilli were also present in large numbers in the necrotic tissue outside of the blood-vessel. They were most numerous around the borders of the areas showing a partial liquefaction. The bacilli were also found singly throughout the fibrinous portions of the thrombi and free in the blood-vessels.

No evidences of tubercle-formation were found in the decidua. Epithelioid and giant-cells were wholly absent in the lesions involving only decidual tissue. In the lowest portion of the basal decidua evidences of such a reaction were seen in the connective-tissue of the uterine wall. About some of the blood-vessels a few giant-cells and a

beginning epithelioid proliferation were seen, but these must be regarded as constituting a uterine tubercle and not a decidual one. Further, in the superficial portion giant-cells occurred in thrombi extending from the decidual sinuses into the intervillous blood-spaces. In this case, the giant-cells, in my opinion, originated from the stroma of chorionic villi involved in the thrombus. The decidual lesions were purely of the nature of a caseous necrosis. In the decidual septa of the placenta areas of tuberculous caseation similar to those in the basal decidua were present. These,

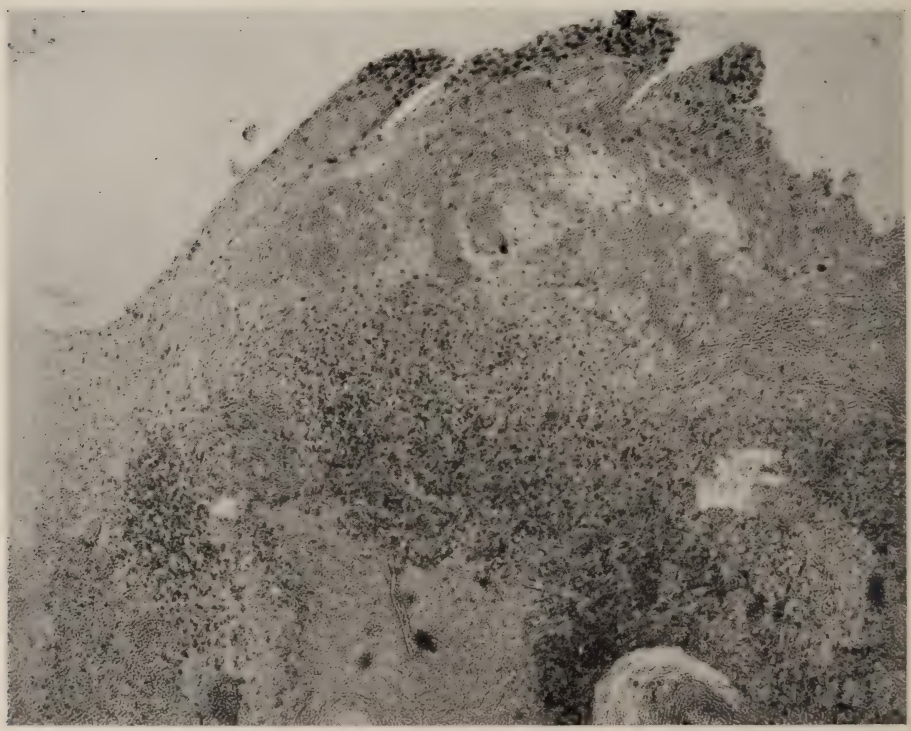

FIG. 3.—Higher power view of portion of FIG. 2; edge of tuberculous necrosis.

likewise, were lacking in epithelioid and giant-cells, except where derived from neighboring chorionic villi. In all cases the necrotic foci were in close association with thrombi in the decidual vessels. The minute study, exhaustive in its thoroughness, that was accorded this case revealed not a trace of a pure decidual tubercle.

Such a phenomenon is, I think, not to be explained upon the ground of a very acute process, since the decidual lesions were very much more marked and apparently older than those of the intervillous spaces and chorionic villi, but in the latter the formation of epitheli-

oid and giant-cells was the rule, even in the smallest lesions. On the contrary, then, the absence of tubercles in the decidua points rather to some individual peculiarity of decidual tissue, in so far as a reaction to infection with tubercle bacilli is concerned. In this one case, at least, under the action of developing bacilli, the decidual cells have

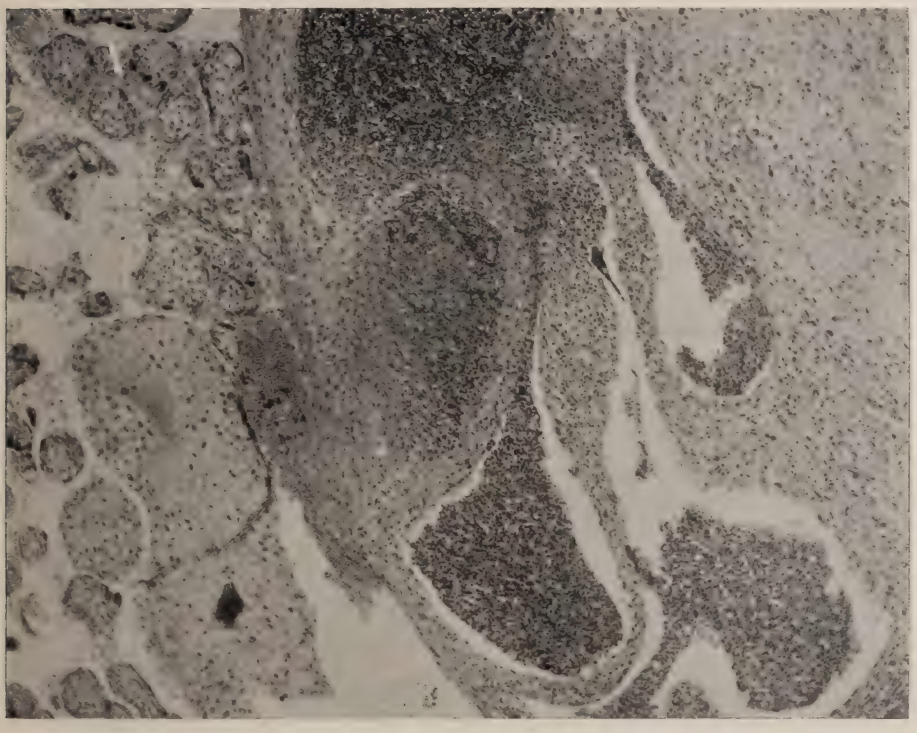

FIG. 4.—Advanced stage of tuberculosis of decidua. Partial liquefaction of tuberculous foci.

undergone necrosis at the point of bacillary growth without the decidual tissue about the necrotic foci showing the slightest signs of a reactive proliferation such as is practically always the rule in tuberculosis of other tissues. It is true that in very acute and virulent cases of miliary tuberculosis the patient may die before the tubercle-reaction has taken place, so that at autopsy the pathological findings are those of focal necroses containing tubercle bacilli. Both factors of acuteness and virulence may be ruled out in this case, since the

much smaller lesions in the fetal portion of the placenta showed true tubercle formation. This leaves us, then, to the conclusion that the decidual cells simply die under the action of tubercle bacilli and that they are not able to form epithelioid cells and giant-cells. The explanation of this we may seek in the more highly differentiated character of the decidual cell as compared with an ordinary connective-tissue cell.

In the second case of placental tuberculosis reported by me a similar condition was seen. The formation of epithelioid and giant-cells was limited to the stroma of the villi; but was not seen in the portions of decidua attached to the placenta. In these the tuberculous lesions were purely necrotic. These two observations may be taken as indications that tubercles are not formed in the decidua. Neither the endothelium of the decidual sinuses and blood-vessels nor the decidual cells themselves appear to have the power of forming epithelioid cells and giant-cells. Further, the absence of giant-cells from the tuberculous lesions in the decidua may be taken also as evidence against the formation of these cells from leukocytes.

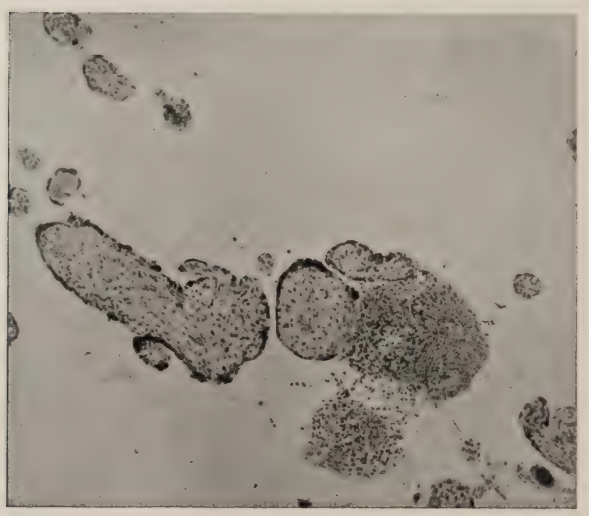

FIG. 5.—Earliest stage of intervillous tubercle. Agglutination-thrombus on chroionic villus. Syncytium is lost at base of thrombus.

Turning to the literature for information as to the nature of decidual tuberculosis, we find that the earlier observations on placental tuberculosis are wholly lacking in this respect. Up to the time of Runge's paper (1903) the only placental lesions described were those affecting the chorionic villi. Lehmann had, it is true, found in his first case a small area of round cells in the decidua which he regarded as tuberculous

but he was unable to demonstrate the presence of any tubercle bacilli. Runge regarded his own case as the first one in which tuberculous lesions of the decidua were definitely shown to exist. His description, so far as it goes, coincides in all respects with that given above of my two cases. In the basal decidua he found sharply circumscribed areas staining more intensely with eosin and hematoxylin than the surrounding tissue. Around the periphery of these round cells in process of karyorrhexis and chromatin fragments were present, while the centers consisted of a finely granular detritus without round cells or nuclear fragments. No epithelioid cells or Langhan's giant-cells were found, although serial sections were made. Staining for tubercle-bacilli showed great numbers of the bacilli in and between the round cells and in the necrotic centers. These lesions were present chiefly in the basal decidua and in greatest numbers on the uterine side of Nitabuch's fibrin band. In a decidual septum extending in between the chorionic villi a similar area was found. In the lowest layer of the decidua in the neighborhood of the uterine glands some resemblance to epithelioid formation was seen.

Runge comments upon the failure of the decidua to form characteristic tubercles of epithelioid cells and giant-cells. Contrasting this absence of reaction with the practically universal formation of tubercles in other tissues of the body, he attempts to explain the phenomenon by the temporary character of the decidua and its slight capacity for proliferation, the latter being shown also in the rarity with which tumors develop in the decidua. That epithelioid cells may be found in the tuberculous lesions in the deepest layer of the decidua bears out the above view, since this portion of the decidua retains to a greater degree its original character and is not destroyed after birth.

In a case of abortion during the fourth month Westenhoeffer found a tuberculous focus in the decidua from which, according to his belief, an acute miliary tuberculosis of the mother developed. While the lesion is not sufficiently described, the changes in the decidua appear to have been essentially the same as those described by Runge.

Schmorl and Geipel found decidual lesions in one of their nine cases of placental tuberculosis. These were of the same character as those described by Runge, consisting of focal necroses containing tubercle bacilli; and lacking the characteristic tubercle formation. Giant-cells were found only in the deepest layers of the decidua.

In Wollstein's case the decidua showed extensive caseation, in some places extending throughout its entire thickness and into the muscle coat. No mention is made of epithelioid or giant-cells, but small tubercles with cheesy centers were found in the uterine musculature. Since no tubercles of the decidua were mentioned, it is fair to conclude that they were not present.

It will be seen, then, that in all of the cases of placenta tuberculosis in which the lesions of the decidua are mentioned, the latter have been of a uniform character, presenting as a caseation necrosis associated with thrombosis and without epithelioid and giant-cell formation. In common with Runge I believe that the failure of decidual tissue to form tubercles can be explained only by the peculiar character of the decidual cells. His explanation, however, does not seem to me to be wholly adequate. More lies behind this phenomenon than the temporary character of the decidua. Accepting the view that decid-

ual cells are transformed stroma cells, in their peculiar transformation the cells have lost the power of responding to ordinary "irritants," in much the same way that the cells of a sarcoma have lost it. In two cases of secondary tuberculous infection of sarcoma seen by the writer the sarcoma-cells had undergone a caseous necrosis without the formation of giant-cells or epithelioid cells. True tubercles were found only in the remains of the original connective tissue of the affected part in one of the cases. In the other they were present only in the connective tissue of the trabeculae, the sarcoma being of the alveolar type. As decidual tissue has sometimes been called a "physiological sarcoma," the analogy is strengthened by the similarity in the absence of a granulomatous reaction to tuberculous infection. My explanation, then, is that the stroma cells in their transformation into decidual cells have already passed into an epithelioid form, and are incapable of further proliferation under the action of such stimuli as tubercle bacilli.

Runge and Schmorl and Geipel regard the thrombosis of the decidual vessels and sinuses as secondary to the tuberculous lesions in the decidua. In my opinion the decidual lesion is pratically always primarily a vascular one, the endothelium being first involved. The hyaline and agglutination thrombi are formed coincidently with, or immediately after, the injury to the endothelium and before the involvement of the neighboring decidual cells. The larger fibrin-masses are formed secondarily. Extensive thrombosis may be the immediate etiological factor of the abortion or premature birth. Congestion of the decidual sinuses (so-called decidual hemorrhages), infarcts of the decidua and chorion, etc., may be caused by the blocking of the placental sinuses.

The tuberculous thrombi are so characteristic that in a section stained with hematoxylin and eosin they readily catch the eye by the contrast they offer to the ordinary thrombus of the placental sinuses. They form the chief diagnostic feature of the microscopical examination for decidual tuberculosis. As their importance has been entirely overlooked by Runge and Schmorl and Geipel, I take this opportunity of emphasizing it. Aside from their serving as an aid to diagnosis, these thrombi are to a certain extent protective in localizing the primary lesion. Tubercle bacilli, however, spread through the

wall of the injured vessel and the necrosis extends into the surrounding tissue. It is, of course, possible that bacilli may pass out into the decidua and give rise to a primary lesion outside of a blood-vessel. Runge believed this to occur, but in my cases the decidual lesions were apparently all primarily vascular.

The thickening of the walls and the obliteration of some of the decidual vessels noted by Runge and believed by him to be inflammatory in character and secondary to the tuberculous process may be found in the normal decidua in the later months of pregnancy. These changes are a part of the physiological maturity and beginning involution of the decidua. When occurring prematurely, as in Runge's case in the fourth month, they are to be regarded as evidences of a premature retrogression. Such changes are especially common in cases of maternal syphilis. Runge thought that these vascular conditions were responsible for the thrombosis seen in his case, but I do not believe this to be the correct interpretation.

The entire absence of giant-cells from the decidual lesions may be regarded as strong proof that they are not formed from lymphocytes. Lymphocytes were numerous in both decidual and chorionic lesions, giant-cells were present only in the latter. If they can be formed from lymphocytes there would be no reason why they should not occur in the decidua. Since they do not here, and since they occur only in association with epithelioid cells, the evidence is, I think, practically conclusive that they arise only from stroma cells. Likewise, the decidual lesions offer proof that epithelioid and giant-cells are not formed from vascular endothelium.

Summing up the chief characteristics of tuberculosis of the decidua, the following conclusions seem of importance:

1. Tuberculosis of the decidua consists primarily in a local lesion of endothelium with the formation at the point of injury of a hyaline or agglutination thrombus.

2. Extension of the process consists in the gradual formation of an area of caseous necrosis, a round-cell infiltration with subsequent karyorrhexis and a formation of fibrin throughout the focus of necrosis.

3. Tuberculosis of the true decidua differs from that of other organs and tissues in the absence of any tubercle-formation. Epithelioid and giant-cells are not formed.

4. The absence of giant-cells from the tuberculous lesions of the decidua may be taken as an argument against their formation from lymphocytes or endothelium.

5. The failure of the decidua to form epithelioid cells may be explained on the ground that the decidual cells in their transformation from stroma cells have lost the power of reacting to ordinary irritants.

B. INTERVILLOUS TUBERCLES.

Throughout the intervillous spaces there were small, round, deeply staining areas, composed of a finely granular or hyaline substance, containing lymphocytes and polymorphonuclear leukocytes in varying stages of disintegration. The majority of these areas were about the size of a small pinhead, or somewhat smaller. They took the eosin stain more heavily than the hyaline fibrin masses found normally in the intervillous spaces; but the fragmentation and diffusion of the chromatin of the nuclei of the leukocytes gave to many of them a bluish or violet tinge. Through their regular outline, deeper staining, and the constant presence of disintegrating leukocytes they attracted the eye at once as presenting features distinct from those of the normal intervillous thrombi. In their general characteristics they resembled in every respect the hyaline thrombi of the decidual vessels. When stained according to Weigert's fibrin method they showed the presence of fibrin, but the greater portion of the hyaline substance was shown by various stains, as well as by the different transition stages found, to consist of agglutinated red-blood cells and blood plates. The staining for tubercle-bacilli with carbol-fuchsin and methylene-blue showed varying numbers of the bacilli in the thrombi, as well as free bacilli in the intervillous blood-spaces.

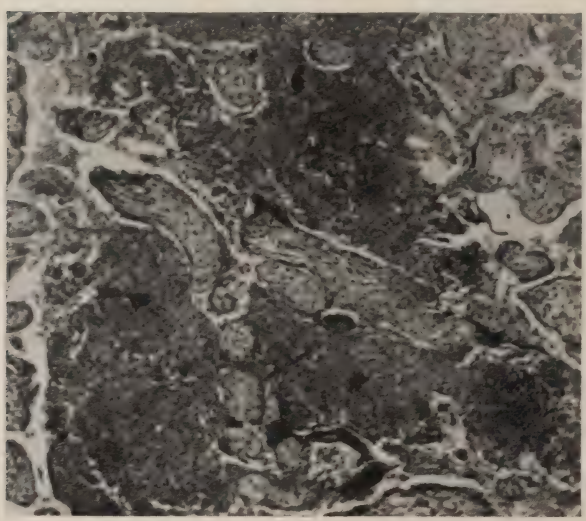

FIG. 6.—Tuberculous agglutination-thrombi in intervillous spaces.

In single sections these tuberculous thrombi often appeared lying between or adjacent to villi covered with syncytium, showing apparently no pathological changes. In other cases they appeared to be lying free in the blood-spaces. Serial sections,

however, showed that in all cases they were attached to a villus at some point where the syncytium had either completely vanished or appeared as a swollen hyaline layer devoid of nuclei. In many cases the necrotic syncytium presented a finely granular appearance, suggesting a beginning caseation. A similar change was seen in the center of many of the thrombi. In those cases in which the syncytium was entirely absent the thrombus rested directly upon the stroma of the villus, which in many instances showed distinct evidences of epithelioid proliferation at the point of contact. Epithelioid cells and typical Langhan's giant-cells were found extending from the stroma of the villus into the thrombus. All stages of this process were found, from thrombi containing a single giant-cell or one or more epithelioid cells to those completely converted into epithelioid tubercles and showing a beginning caseation. In other words, the thrombi were being organized by epithelioid tissue arising from the stroma of the villi, and were thus changed into typical tubercles. Giant-cells were very numerous and very large. In some cases they could be seen extending out into the thrombus while still attached to the stroma of the villus. In many thrombi the only evidence of epithelioid change was found in solitary giant-cells; and these, though often apparently occupying the center of the thrombus had long

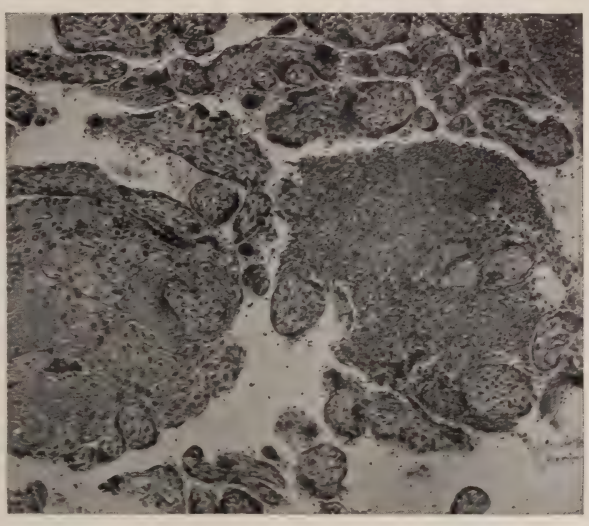

FIG. 7.—Intervillous tuberculous thrombus. First step in formation of tubercle. Giant-cell arising from stroma of small villus that is nearly wholly denuded of its syncytial covering.

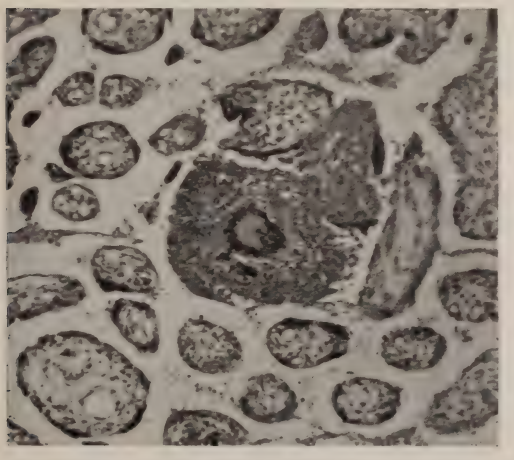

FIG. 8.—Intervillous aggultination-thrombus attached to villus where syncytium is lost. Contains one large giant-cell.

protoplasmic processes continuous with the stroma of the villus. In those thrombi resting upon an intact necrotic syncytium no giant-cells or epithelioid cells could be found. No connection could ever be demonstrated between the syncytium and a giant-cell, and not the slightest evidence could be obtained tending to prove the yncytial origin of the latter. Likewise, no evidence could be found of the origin of the epithelioid or giant-cells from lymphocytes. *On the other hand the evidence seems conclusive that they have a common origin in the stroma of the villi.*

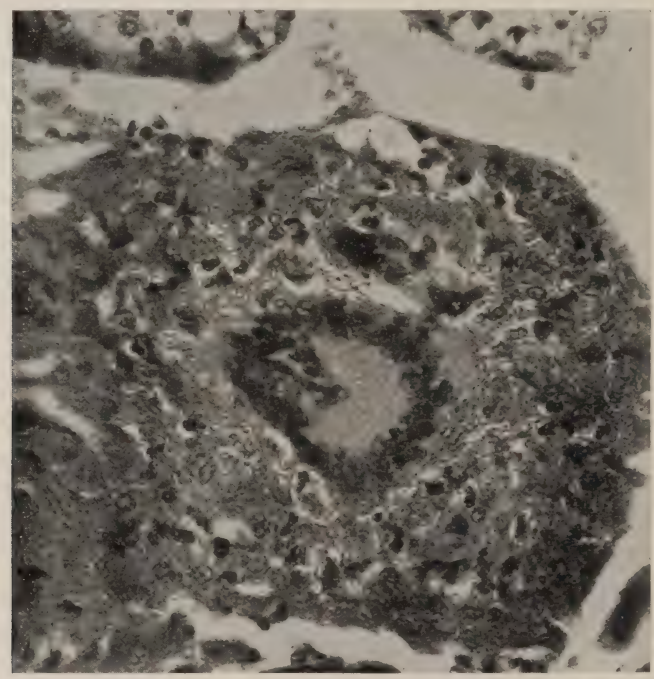

FIG. 9.—High-power view of giant-cell shown in Fig. 8.

The histogenesis of the intervillous tubercles may, therefore, be concisely stated as follows: There is first a lesion of the syncytium of the nature of a degeneration or necrosis, produced by the action of the tubercle bacilli lodging upon the surface of the villus. Upon the damaged syncytium there is deposited from the maternal blood a collection of leukocytes, red-blood cells, and blood-plates, giving rise to a hyaline or agglutination-thrombus. The next step is the epithelioid organization of the thrombus from the stroma of the villus, and the tubercle thus formed later suffers caseation. It is worthy of remark

that the caseous process usually does not begin in the center of the thrombus or tubercle but adjacent to the villus, thus indicating that this is the older part of the tubercle.

In my former paper I expressed the view that the syncytium appeared to possess some resistance to the action of the tubercle bacillus, since some of the thrombi appeared to rest upon an apparently normal syncytium. The study of serial sections since made has demonstrated the occurrence of syncytial lesions in all such cases,

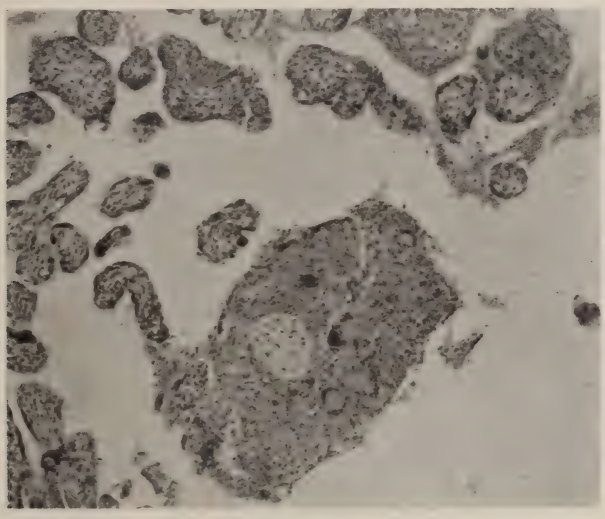

FIG. 10.—Full development of intervillous tubercle. Thrombus nearly wholly organized by epithelioid and giant-cells arising from stroma of villus. Syncytium wholly lost.

and I am now forced to the conclusion that the syncytium does not show any greater resistance to the action of tubercle bacilli than does the vascular endothelium in any part of the body. *The placenta has no especial protection against tuberculosis. In the event of tubercle bacilli gaining entrance to the maternal blood-stream the chances in favor of a placental localization are as great as those of any other organ.* In fact, it is a question as to whether the chances of placental infection are not greater, the large size of the placental blood-spaces and the slowness of the circulation probably favoring the deposit of bacilli carried in the blood.

In the article written with Kockel, Schmorl expressed the view that the epithelioid cells might arise from the chorionic epithelium. In his more recent article with Geipel he has abandoned this view, and now believes that they arise either from the lymphocytes or from the fixed cells of the stroma of the villi. He was, however, unable to decide definitely as to their origin. He regards it as strange that the granulation-tissue, in case it does arise from the stroma, does not extend into the substance of the villi, but, on the contrary, grows outward, sometimes appearing to surround a villus that preserves still its original outlines. Such an outward growth does not always occur alone; many villi showed also an epithelioid ingrowth into the villus. Serial sections show, also, that the epithelioid tissue surrounding such a villus may arise from the stroma at some point either below or above the plane in which the given section was cut, or may have originated in the stroma of a neighboring villus. The outward growth is easily explained by the fact that the developing colony of tubercle bacilli is outside of the villus in the thrombus, and the conversion of the thrombus into a tubercle by an outward growth from the stroma of the villus is carried out according to the laws of chemotaxis and organization.

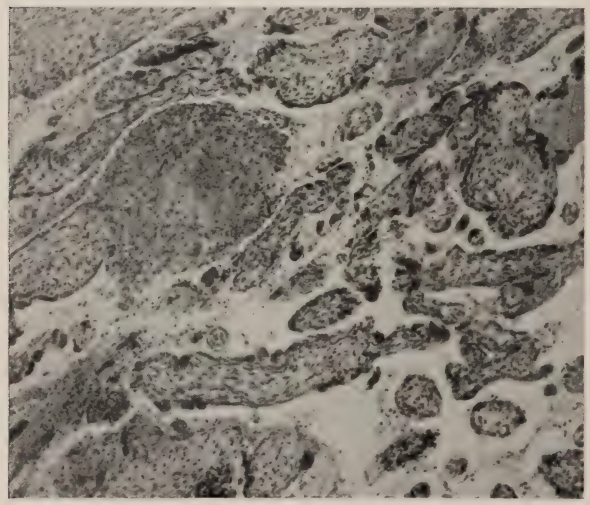

FIG. 11.—Intervillous tubercle with beginning caseation. Note continuity with stroma of villus at point of attachment.

While not absolutely denying the possibility of an origin of epithelioid cells from the lymphocytes included in the tuberculous thrombus, my cases show nothing to support such a view, but, on the contrary,

all appearances favor their origin from the stroma-cells. The fact that many of the thrombi contained in their centers one large giant-cell apparently having no connection with the villus led me, in my first paper, to believe that giant-cells might arise from the white cells included in the thrombus, but my further studies favor wholly their connective-tissue origin. It may be asked if the lymphocytes in the intervillous thrombi have the power of producing epithelioid cells, why have not those found in the thrombi of the decidual vessels? The conditions are the same; but in one case we have an abundant formation of epithelioid cells, even in the smallest sized thrombi, while in the decidual thrombi such a formation is wholly wanting. This phenomenon, to my mind, is a very strong argument against the lymphocytes as a source of epithelioid cells in the formation of placental tubercles.

Conclusions.—The intervillous tubercles are the result of a primary lesion of the syncytium, caused by the growth of tubercle bacilli upon the surface of the villus. At the point of injury there is formed an agglutination thrombus which undergoes an epithelioid organization from the stroma of the villus, and the tubercle thus formed suffers caseation.

There is no evidence that epithelioid or giant-cells are formed either from the syncytium or from lymphocytes. All the findings favor their origin from the connective-tissue of the villi.

C. INTRAVILLOUS TUBERCLES.

In many villi tubercles were found arising in the stroma of the villus independently of any lesion of the syncytium or of any intervillous thrombosis. These intravillous lesions varied from a single giant-cell to epithelioid tubercles involving a part or nearly the whole of the villus and showing more or less advanced caseation. Caseous foci without epithelioid cells also occurred in the stroma of some of the villi. All the caseous areas contained tubercle-bacilli, and these were also found free in the spaces of the stroma of villi showing no pathological changes whatsoever. Even in villi whose stroma had for the greater part been replaced by epithelioid cells the syncytium showed no change. Only when the caseation had reached the subsyncytial layer of stroma did the syncytium become necrotic, and coincident with this change there always occurred a thrombosis at the site of the injured chorionic covering.

The presence of intravillous tubercles in the absence of syncytial lesions and thrombus-formation must, I think, be taken as strong evidence that the bacilli have passed through the syncytium without damaging it, and have set up their characteristic changes first in the

stroma of the villus. The absence of thrombi upon the syncytium must be accepted as indicating that the syncytium is not involved,

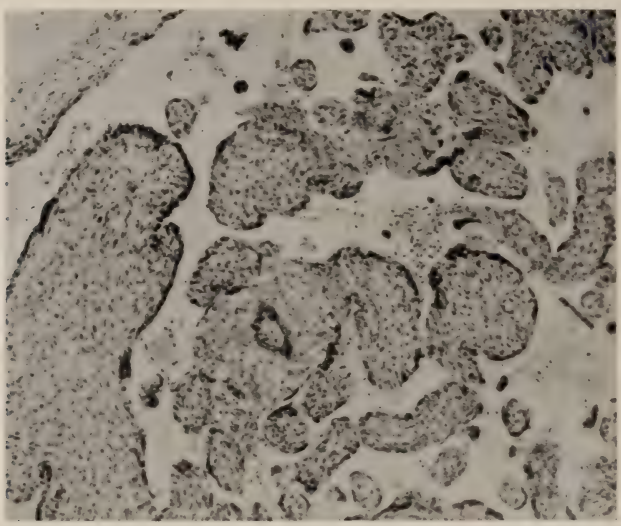

FIG. 12.—Intravillous tubercle. Giant-cell in interior of villus. No thrombosis

or, at least, not to any perceptible extent. The question of main importance here is the route by which the tubercle bacilli have reached the stroma-spaces. They must either have penetrated the syncytium without damaging it, or their presence in the villi may be the result of a retrograde metastasis from some other lesion involving chorionic vessels and liberating bacilli into the fetal circulation.

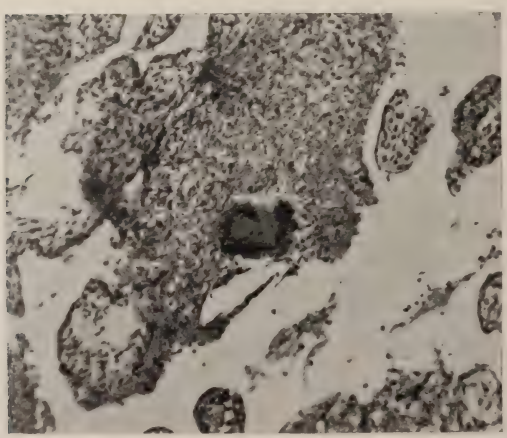

FIG. 13.—Intravillous tubercle. Giant-cell beginning caseation.

Schmorl regards the intravillous tubercle as very rare, having seen it but once in his set of cases. He considers two explanations, an

entrance of bacilli through a defect in the syncytium or an infection from the fetal stream, either through direct metastasis after passing through the fetal body or through a retrograde transportation. Although not so numerous as the intervillous tubercles the intravillous lesions were common in my case. As described above, their character is essentially the same as that of the intervillous. There is first a localized necrosis, followed by an epithelioid proliferation of

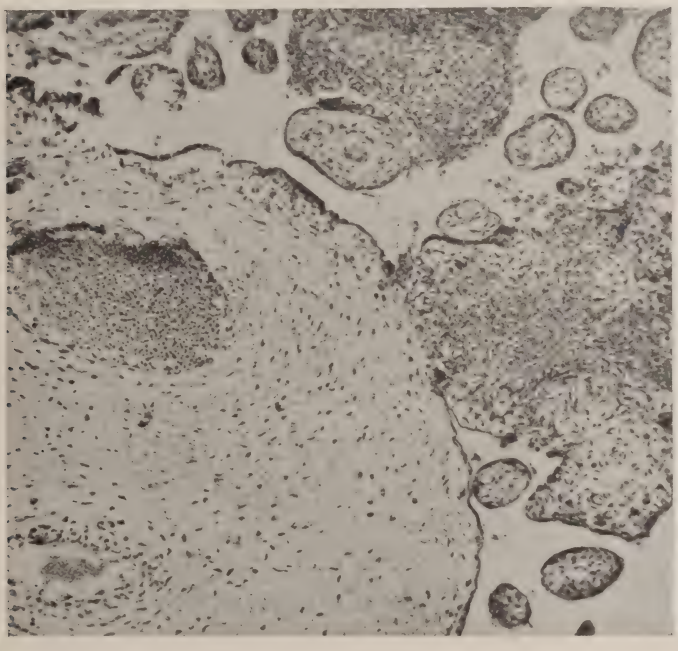

FIG. 14.—Intravillous tubercles with caseation.

the neighboring fixed cells, forming a tubercle which eventually caseates. From the intervillous tubercle they are usually easily distinguished by the absence of a thrombus or by its minor prominence as compared with the changes in the villus itself. While it is not possible absolutely to decide the question, the fact that some villi develop tubercles in their stroma without showing in serial sections any evidence of a syncytial lesion inclines me to the belief that bacilli may pass the syncytium without injuring it. In the absence of

tubercles in the organs of the fetal body, it does not seem likely that the chorionic villi would alone show tubercles if the dissemination occurred by metastasis direct through the fetal blood-stream. A retrograde transportation seems still less likely.

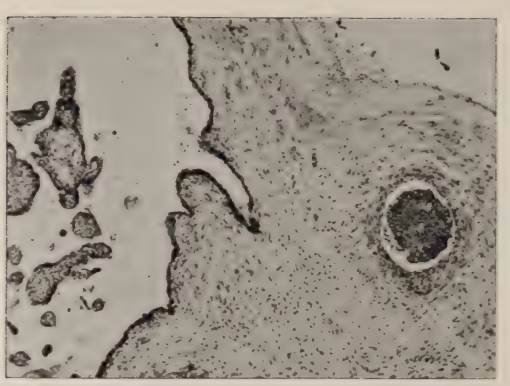

FIG. 15.—Intravascular chorionic tubercle. Agglutination-thrombus in chorionic vessel. Showed beginning epithelioid organization.

Conclusion.—Intravillous tubercles are less common than the intervillous ones. They may occur without apparent lesion of the syncytium and without the occurrence of thrombus-formation on the surface of the villi. It is, therefore, probable that tubercle-bacilli can pass the chorionic epithelium, leaving it unhurt, and set up tubercles within the substance of the villi.

D. INTRAVASCULAR CHORIONIC TUBERCLES.

Of still rarer occurrence were tubercles and tuberculous thrombi in the vessels of the chorion. The thrombi were either obturating or parietal, and were in all respects like those of the intervillous spaces. Attached to the intima of some of the larger chorionic vessels there were deeply staining hyaline or granular masses containing chromatin fragments. At the point of attachment to the wall the endothelium and subendothelial layer showed beginning necrosis. Tubercle bacilli were demonstrated in these thrombi. In the development of a tubercle the epithelioid cells arose in the vessel-wall.

E. CHORIO-AMNIOTIC TUBERCLES.

Secondary involvement of the amnion by large caseating or epithelioid tubercles of the chorion was seen a few times. The portion of the amnion lying in the neighborhood of the chorionic tubercle was thickened, infiltrated with lymphocytes, and showed a beginning

caseation. Tubercle bacilli were contained in the caseous area. Similar lesions were twice found by Schmorl.

SUMMARY.

1. Tuberculosis of the placenta manifests itself in five forms, according to location: (1) Decidual; (2) Intervillous; (3) Intravillous; (4) Intravascular chorionic; (5) Chorio-amniotic.

2. In all forms the primary lesion is a *necrosis* of the cells at the point of bacillary growth, either upon the syncytium or endothelium, or in the decidua or stroma of the villi.

3. Lesions of syncytium or endothelium lead to the formation of hyaline or agglutination thrombi. These may become converted into tubercles through an epithelioid proliferation of neighboring fixed cells, and the tubercles so formed become caseous.

4. Decidual tuberculosis is characterized by necrosis of the decidual tissue and thrombosis of the sinuses without the formation of giant- or epithelioid-cells. Tubercles are not formed from the decidual cells.

5. The syncytium of the chorionic villi is no more immune to the action of tubercle bacilli than is the vascular endothelium in any part of the body.

6. There is some ground for the belief that tubercle bacilli may pass through the syncytium without causing injury to it.

7. The evidence afforded is against the formation of giant-cells from lymphocytes, endothelium, or syncytium but is in favor of their origin from connective-tissue cells.

In conclusion, the histological study of placental tuberculosis throws important light upon the general nature of the primary lesion produced by tubercle bacilli. The view advocated by Baumgartner and others that the tubercle bacillus causes primarily a cell-proliferation is flatly contradicted by the findings in tuberculosis of the placenta. Here it is clear that the primary lesion is in all cases a *necrosis* of cells at the point of bacillary development. The formation of a tubercle through a secondary cell-proliferation of connective-tissue cells immediately about the primary necrosis occurs wherever there are connective-tissue cells at the point of primary necrosis. In the case of the decidua the tuberculous lesion never gets beyond

the stage of primary necrosis, cell-proliferation not taking place, so that decidual tubercles are not formed. The tubercle is, therefore, a secondary formation, inflammatory in nature, and reparative and protective in function.

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LEUKEMIA OF THE COMMON FOWL.*

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LEUKEMIA appears to be of rather frequent occurrence in the domestic animals. The statistics of Nocard (1880–82) comprised twenty-two cases in the dog, nine in the horse, six in the hog, five in the cow, and one in the cat. Since 1891 a number of new cases of leukemia in cattle, horses, dogs, and swine have been observed, and the disease has been shown to occur also, though less frequently, in the goat, sheep, and mouse. In the case of the rabbit and guinea-pig, the animals most frequently used in experimental work, there exist at present no observations of the occurrence of leukemia. The literature is likewise devoid of any statements concerning its occurrence in birds and fowls.

Moore, in 1895–96, reported observations upon an epidemic disease of fowls under the designation of "infectious leukemia." From the blood of diseased fowls showing a great increase in the total number of white cells he isolated *B. sanguinarium*, and regarded this as the etiological agent. There can be no doubt that the condition described by Moore was not one of true leukemia, but only a marked leukocytosis due to a bacillary infection. The increase in the white cells appears from his description to have been limited to the crystalloid eosinophile cells. No atypical mononuclear cells are mentioned, and the general pathological picture is that of an infectious disease and not that of leukemia. The cellular infiltrations of spleen and liver so characteristic of the leukemias were wanting, and the very short course of the process speaks for the non-leukemic nature of the infection. Moore's cases must, I think, be rejected as instances of avian leukemia.

Butterfield, in 1905, reported from this laboratory three cases of aleukemic lymphadenoid tumors of the hen. In all three cases attention had been directed to the very marked enlargement of the liver, the material having been sent to me for examination because of this feature. The histological examination of the livers of the three cases showed the same condition in all. The liver tissue was nearly wholly

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replaced by masses of cells resembling in general the large lymphocytes of the hen's blood, although showing slight individual variations. With the exception of slight changes due to pressure, these cells were round or oval, and possessed round or oval nuclei, usually placed somewhat eccentrically. The chromatin was reticular or granular, and the narrow rim of protoplasm nongranular and basophilic. Between the cells there was a delicate stroma. Evidences of rapid growth were found in the presence of numerous mitoses. The small amount of liver tissue remaining consisted of strands or islands of liver cells lying in or between the masses of lymphoid cells, the liver cells showing the effects of compression conforming to the shape of the tumor areas. The latter were oriented as being periportal, the smaller groups of tumor cells surrounded by hepatic cells as being direct outgrowths of the periportal masses in the great majority of instances, although apparently metastatic in others. At the borders of the tumor masses great numbers of crystalloid eosinophiles, mostly mononuclears, were seen. No increase of white cells was found in the blood-vessels. The type of cell, the periportal growth of the tumor masses, and the absence of any increase of white cells in the circulation led to the diagnosis of *aleukemic lymphadenoma*, or, according to the terminology proposed by me as *aleukemic leukoblastoma* of the lymphocyte type (*lymphocytoma*).

The establishment of the existence of an aleukemic lymphocytoma in the fowl led me to believe that the leukemic form must also exist and that its demonstration was only a question of chance and material. Arrangements were made by which fowls showing symptoms of disease other than the common poultry affections would be brought to the laboratory for examination. Dr. Butterfield in the meantime, had learned through correspondence with Dr. Mohler of the Bureau of Animal Industry that the latter had examined the carcasses of five fowls showing a condition of disseminated lymphadenoma with an increase of the lymphocytes in the blood-vessels. Through the courtesy of Dr. Mohler slides were sent to us for examination, and from these there could be no doubt that the condition was one of leukemic lymphocytoma. The tumor-like infiltrations of cells presented the same histological features as in our cases, but in addition the blood-vessels contained great

numbers of white cells, the predominating type being large lymphocytes precisely like the cells of the tumor-like infiltrations in both his cases and ours. As Dr. Mohler had unfortunately examined only the carcasses, no blood-counts or differential counts in stained smears were made, and for this reason these cases were not reported, Dr. Mohler awaiting an opportunity of studying the condition in the living fowl. To my knowledge this has not yet been done; but an opportunity is here taken of crediting Dr. Mohler with the observations made by him.

In December, 1905, there came into my hands a Buff Cochin Bantam hen showing signs of illness in the way of indisposition to move about and a general weakness of a progressive character. No symptoms of the ordinary fowl diseases were present. Catarrhal conditions were entirely absent. As a number of fowls presenting similar symptoms had been examined and found to have avian tuberculosis, it was thought at first sight that this fowl was similarly affected. Examination of blood-smears showed, however, a great increase of white cells of the large lymphocyte type, instead of the crystalloid eosinophile leukocytosis found in the tuberculous hens. A diagnosis of leukemia was therefore made, and thorough blood-examinations were carried out until the death of the fowl, which took place about two and one-half months after it was first seen. During this time the hen showed a progressive weakness and emaciation, with periods of apparent improvement. The comb lost its red color, becoming gray, the yellow portions of the legs also became gray. Much of the time the hen maintained a sitting posture, and when disturbed would move a few steps and then sit down. Food was taken in very small amounts except during the periods of apparent improvement. For a few days before death thin watery fecal discharges were noted. Death resulted from increasing weakness, the fowl dying while a blood-count was being taken.

BLOOD.

The blood for counting and for smears was obtained from cuts made into the comb with a sharp knife. The slight wounds thus made bled abundantly. Toison's solution was found to be the most satisfactory for the blood-counting. Hematoxylin and eosin, Ehrlich's triple stain and Wright's method were used for staining the smears. The best results, on the whole, were obtained by Wright's, when this stain was used immediately after the smears were made. When used with smears that had been

allowed to dry in the air for some time before staining, both red and white cells showed marked degeneration and stained poorly.

Blood-count.—Red-blood cells = 450,000; white cells = 280,000. This gives a proportion of less than two red cells to a white cell. While considerable variation in the number of white and red cells was found at different times, the above represents the average count.

Stained smears.—These showed the great increase of white cells found by the blood-count, and numerous differential counts were made. While showing some variation in the smears made at different times the average differential count, 500 white cells, was as follows:

Small lymphocytes	1.5	per cent
Large lymphocytes	84.5	"
Granular eosinophiles	0.0	"
Crystalloid eosinophiles	11.5	"
Degenerating white cells	2.0	"
Mast cells	0.5	"
	100.0	"

In some counts the proportion of large lymphocytes was found as high as 90 per cent, in others as low as 80 per cent. These cells varied greatly in size, all transition-stages being found between those of normal size and those several times as large. The protoplasm was basophilic and usually non-granular, and varied greatly in amount, from a small rim around the nucleus to a band exceeding in width the diameter of the nucleus. The latter stained a clear light blue in the smaller forms of the large lymphocytes, in the large ones a decided reddish violet. In the majority of cells the nucleus was placed somewhat eccentrically. It was reticular or granular, and in many cells vacuolated. In the larger cells with nuclei staining violet, the chromatin was much more reticular than in the smaller cells. These cells also contained more and larger vacuoles, and are probably to be regarded as degenerating lymphocytes. No evidence of any intra-cellular parasite or inclusion could be found in the lymphocytes. No division figures were found. The small lymphocytes were greatly reduced in number, as were also the eosinophile cells. Of the latter, numerous mononuclear forms were found. The mast cells were not increased. The red-blood cells were pale, and showed marked poikilocytosis. The labile cells were diminished in number, and many atypical forms of these were seen, some containing two nuclei.

As a control, counts and stained smears were made of the blood from healthy fowls of the same variety. Considerable variation was found in the number of both red and white cells under apparently normal conditions. The average count may be given as follows:

Normal Hen's (Buff Cochin Bantam) red cells = 2,000,000–3,000,000.
" " " " " white cells = 12,000–29,000.

A proportion of 105–225 red cells to one white cell obtains.

DIFFERENTIAL COUNT.

Small lymphocytes	35.5	per cent
Large "	14.5	"
Granular eosinophiles	10.0	"
Crystalloid "	21.5	"
Degenerates	16.5	"
Mast cells	2.0	"
	100.0	"

When compared with the blood of normal hens of the same variety the blood of the diseased fowl presented the following differences:

1. Great reduction in the total number of red-blood cells, only one-sixth to one-eighth of the normal number being present. These showed great variation in size and shape, and the haemoglobin was much reduced. The labile cells were diminished in number and evidences of nuclear degeneration were found in many of them.

2. A great increase in the total number of white cells, so that a proportion of one white cell to two reds obtained. Further, a marked disturbance in the relative proportion of the different types of white cells, the polymorphonuclear cells and small lymphocytes being replaced to a large extent by cells of the large lymphocyte type. Numerous atypical mononuclear leukocytes were also present in the blood.

From the blood-changes a diagnosis of *lymphatic leukemia* of the large-cell type seemed justified and was accordingly made.

AUTOPSY PROTOCOL.

The autopsy of the fowl was made immediately after its death, while the tissues were still warm.

Small, emaciated fowl. Total weight 450 grms. Comb and wattles very anemic, grayish in color. No fat in body. Small amount of pale blood present in heart and blood-vessels. All tissues pale and anemic. Muscles very atrophic.

Liver.—Weight 34 grms., about $\frac{1}{13}$th of the entire body weight. Greatly increased in size: five or six times that of the normal Bantam liver of same variety. It filled up the greater part of the abdominal cavity. The general shape was fairly well preserved; somewhat more spherical than normal. Color was pale yellow; uniform surface; consistency soft, crumbling, very easily torn. No liver structure was apparent. Scrapings yielded abundant cells of type of large lymphocyte, in all respects identical with those of the circulating blood.

Spleen.—Enlarged; spherical; Weight 2.5 grms; reddish-yellow in color; soft and friable. On section, presented numerous yellowish spots about the size of a pinhead.

Heart.—Weight 3.5 grms; dilated; muscle pale, thin, and soft. Contained a few small yellowish points in the muscle.

Kidneys.—Weight 2.0 grms. each; enlarged. Throughout the kidney substance there were numerous small, yellowish spots, varying in size from that of a pin-point to that of a mustard-seed.

Hemolymph Nodes.—Two large reddish-yellow masses lay along the median line of the backbone. Weight 2.5 grms. each. They also contained small yellowish nodules.

Bone-marrow.—From the femurs solid plugs of reddish-yellow, friable marrow were obtained.

With the exception of atrophy and anemia the other organs and tissues presented no pathological appearances.

MICROSCOPICAL EXAMINATION.

Preparation.—Smears were made of the blood and from the cut surfaces of all the organs and tissues. Part of these were fixed in alcohol and ether and stained with hematoxylin and eosin, Ehrlich's triple stain, etc. The others were stained by Wright's method. Portions of the organs and tissues were fixed in mercuric chloride, Zenker's, 4 per cent formaldehyde, and Flemming's solution. They were imbedded in paraffin and stained in various ways.

Smears.—The smears of blood taken at the autopsy resembled those made during life, with the exception that the number of degenerating white cells was greatly increased. The smears made from the liver, spleen, and bone-marrow showed a preponderance of cells of the large lymphocyte type, identical with those in the circulating blood.

Liver.—Sections of this organ showed the liver-tissue to be almost entirely replaced by masses of small, round cells, corresponding in type to the large lymphocytes found in the circulating blood. Between these cell-masses there were present strands and islands of liver cells in varying stages of atrophy, cloudy swelling, and fatty degeneration. The capillaries between the liver-rods were filled with white cells, in great excess over the red cells. As no portal structures were found in the areas of hepatic tissue, and as these could occasionally be seen in the masses of lymphoid cells, the latter were oriented as periportal infiltrations and proliferations. Smaller islands of lymphoid cells apparently surrounded by hepatic cells were found to be continuous with each other and with the larger masses. The appearance of multiple tumor-nodules shown by a single section is explained by serial sections as resulting from such outgrowths into the lobules from the main periportal masses. Staining with Mallory's reticulum stain showed that each large nodule had for its center a periportal tract.

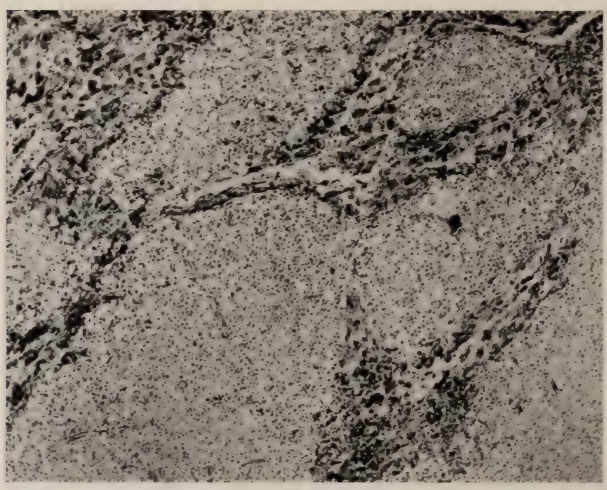

FIG. 1.—Fowl's liver in leukemia. Cells of large lymphocyte type arranged in periportal tumor-like masses infiltrating and destroying the liver-tissue. Leukemic infiltration.

The cells of the periportal infiltrations showed slight individual variation. They were round or oval, and as a rule possessed pale, round, or oval nuclei, placed somewhat eccentrically. The chromatin was granular or reticular. Vesicular nuclei with deeply staining nucleoli were often seen. The basophilic protoplasm formed a ring of varying width about the nuclei. Numerous mitotic figures, apparently nor-

mal, were seen throughout the cell masses. No multinuclear or giant cells were seen. Between the cells there could be demonstrated a delicate stroma. No retrograde changes were found in any of the areas of lymphoid cells. In some nodules the lymphocytes were packed more densely around the periphery, giving an appearance resembling that of a germ-center. In the peripheral zone of the lymphoid collections bordering upon the hepatic cells great numbers of crystalloid eosinophiles were seen, the majority of them being mononuclears. The larger blood-vessels of the liver showed an excess of white cells over red, the great majority of the former corresponding to the cells of the periportal masses and to the type of the large lymphocytes of the blood-smears.

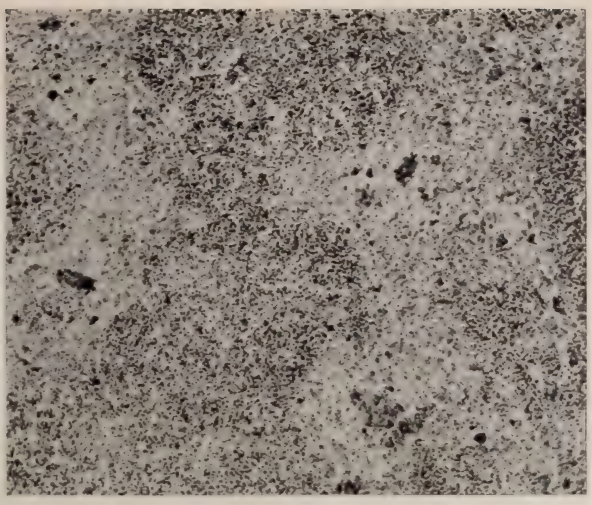

FIG. 2.—Fowl's liver in leukemia. Parenchyma nearly wholly replaced by lymphoid growths.

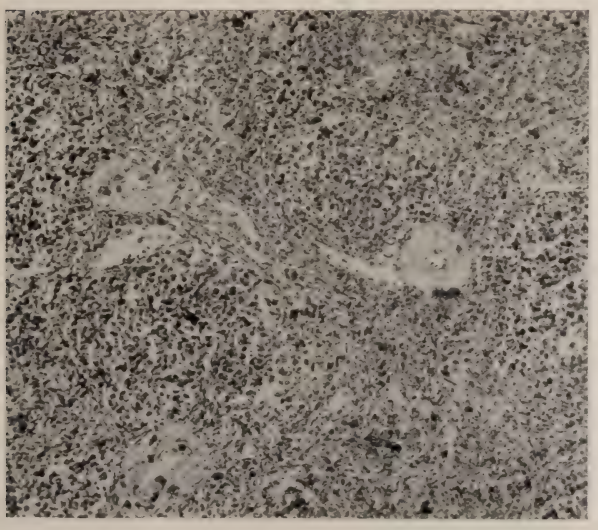

FIG. 3.—Fowl's spleen in leukemia Normal spleen tissue replaced by cells of large lymphocyte type, sometimes arranged in masses suggesting centers of proliferation. The stroma is also increased.

Spleen.— Sections of the spleen showed a marked hyperplasia of the stroma and a replacement of normal spleen tissue by an atypical lymphoid tissue similar to that found in the liver. The yellowish points consisted of collections of lymphocytes apparently in more rapid proliferation, the cells being crowded densely around the periphery of a lighter-staining

central area. Eosinophiles were not so numerous as in the liver, and had no definite arrangement. The same excess of white cells was found in the larger vessels as that seen in the hepatic blood-vessels.

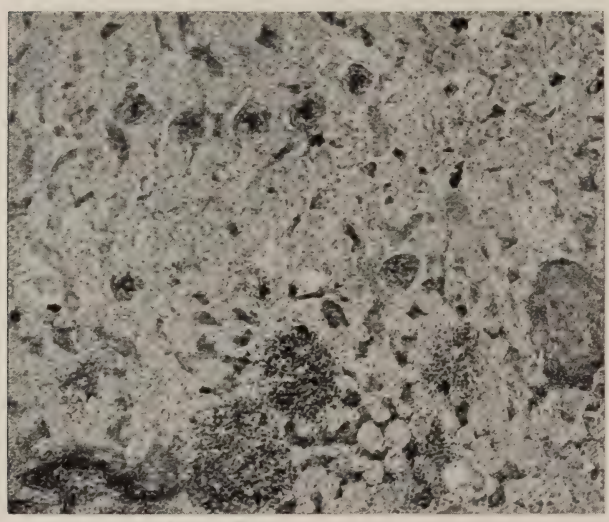

FIG. 4.—Kidney of leukemic fowl. The glomeruli show various stages of lymphocyte embolism and proliferation interpreted as leukemic metastases.

Heart. — Sections of this organ showed an excess of white cells of the large lymphocyte type in the blood-vessels, and a few small perivascular infiltrations of cells of the same type as those in the liver and spleen. The muscle was atrophic and showed slight fatty degeneration.

Kidneys.–Throughout both kidneys numerous small nodules of lymphoid cells occurred, varying greatly in size, but made up of the same type of large lymphocyte found in the blood and in the infiltrations in the liver and spleen. These nodules occupied the sites of glomeruli, and all transition stages of development could be seen, from glomeruli containing a small collection of these cells in the glomerular capillaries, suggesting an embolus, to larger masses completely destroying and replacing the glomeruli. About the larger nodules the kidney structures were compressed or destroyed, and cords of lymphoid cells infiltrated out from the main mass into the surrounding renal tissue, in all respects after the manner of

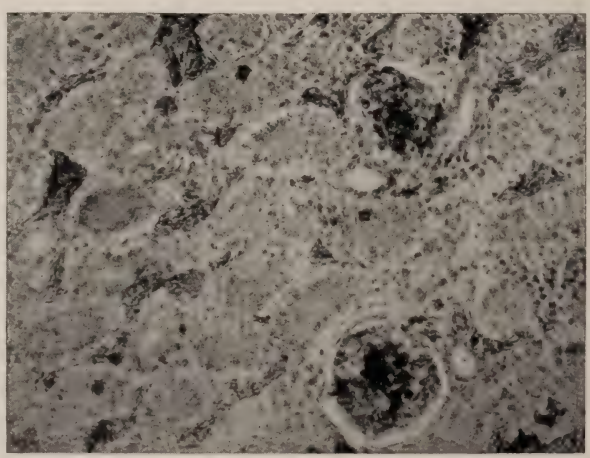

FIG. 5.—High-power view of preceding showing collections of lymphocytes in glomerular capillaries.

a malignant tumor. Not all glomeruli contained cell-masses; some were entirely free from any lymphocyte collections. Others showed only an excess of white cells in the capillaries without any apparent embolism or any proliferation. On the whole the appearances give the impression that the nodules of lymphoid cells arising in the glomeruli are of the nature of tumor-metastases arising from emboli of lymphoid cells in the glomerular capillaries. Mitotic figures were common in these nodules. Their infiltrative and destructive action upon the surrounding kidney structures is in all ways analogous to that seen in the case of malignant tumors. The blood-vessels of the kidney showed a great excess of white cells over red.

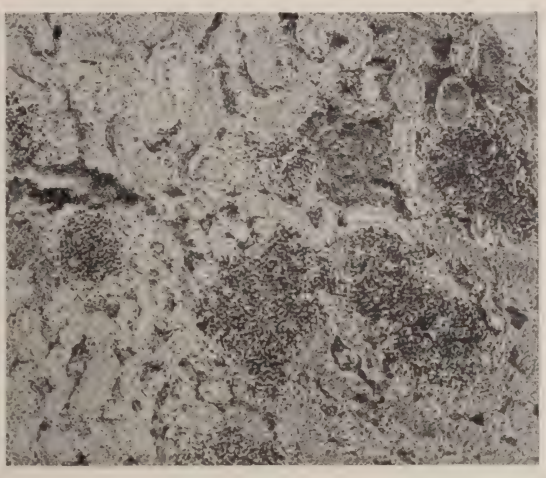

FIG. 6.—Leukemic metastases and infiltration of fowl's kidney.

Bone-marrow.—The bone-marrow appeared to consist chiefly of cells of the large lymphocyte type, the number of red-blood cells, eosinophiles, and mast cells being greatly diminished. No giant or multinuclear cells were found. Throughout the marrow there were numerous small areas having a lighter center and a densely crowded periphery, resembling germ-centers. The cells composing these areas were of the large lymphocyte type, similar in all respects to the cells of the tumor-nodules and infiltrations in

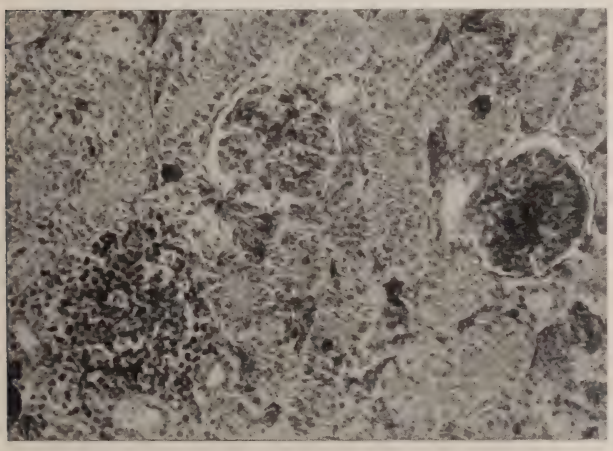

FIG. 7.—Kidney of leukemic fowl. Three glomeruli in various stages of lymphocyte infiltration. One glomerulus is practically normal, a second one contains a lymphocyte embolus with beginning proliferation, while in the third the growth of the lymphocytes has destroyed the glomerulus and there is an inital infiltration of the surrounding renal tissue.

the liver, spleen, and kidneys. The changes in the marrow were interpreted as being of the nature of a lymphoid hyperplasia replacing the red-cell-forming tissue. The areas resembling germ-centers might be explained as metastases or as centers of more active proliferation.

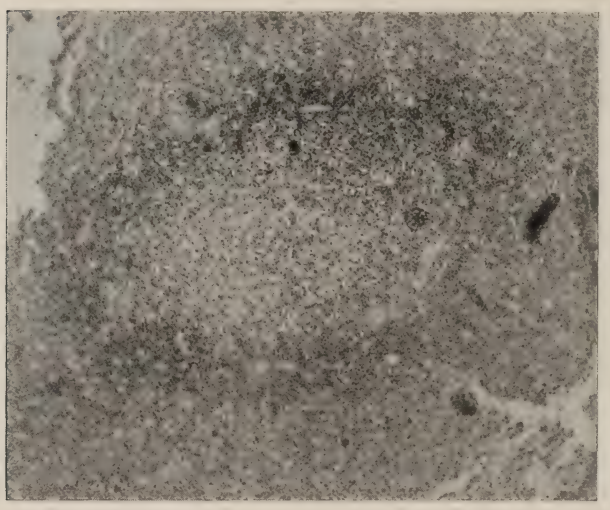

Fig. 8.— Bone-marrow of leukemic fowl. The light area with darker border represents either a primary center of lymphocyte proliferation or may be interpreted as a metastasis.

Hemolymph nodes.—The reddish masses lying along the backbone were shown microscopically to be made up of collections of lymphoid cells and blood-sinuses. The type of cell was that found elsewhere, as described above. The blood-vessels and sinuses contained a great excess of white cells of the large lymphocyte type.

The examination of the remaining organs and tissues showed throughout the leukemic blood-picture in all the blood-vessels with the occurrence of extra-vascular collections of lymphocytes. The parenchyma of all the organs exhibited more or less atrophy and fatty change.

The tissue changes may, then, be summarized as follows:

1. Tumor-like nodules and infiltration of lymphoid cells in all the organs, particularly in the liver, spleen, kidneys, bone-marrow, and hemolymph nodes.

2. Replacement of the normal white cells of the blood by cells of large lymphocyte type, more or less atypical in character but identical with the cells of the tumor-masses.

3. Secondary atrophy and degeneration.

DIAGNOSIS.

The findings, both in the blood and in the tissues, fully warrant the diagnosis of *leukemic lymphocytoma* (large-celled lymphemia).

ETIOLOGY.

All attempts to discover an etiological agent failed. The examina-

tion of the blood and tissues for bacterial and protozoan parasites was thoroughly carried out and gave absolutely negative results. A great variety of staining methods were used, including the most recent methods for the staining of spirochetes and protozoan parasites. Nothing suggesting a parasite was found. No inclusions of any kind were seen in the white cells. Cultures upon a variety of media were sterile, both aerobic and anaerobic. No evidence of the existence of any infective agent could be obtained. The plans for inoculation and transplantation miscarried in this case. They are being prosecuted in the aleukemic variety of lymphocytoma as new material is obtained. It may be mentioned in this connection that Weil and Clerc failed to obtain any results from the inoculation of blood and the transplantation of portions of lymphadenoid tumors in the case of leukemia of the dog.

Since the above case was studied two new cases of lymphadenoid tumors in the common fowl have been received at the laboratory, the material having been sent in by the health officer of Ann Arbor, Dr. Wessinger, for examination for suspected tuberculosis. One of these cases is of the aleukemic condition, as described by Dr. Butterfield; the other is of the leukemic form, as described above. The histological picture apart from the increase of white cells in the blood vessels in the one case, is identical in the two cases and also with that in the cases given above. From these cases we must conclude, therefore, that there occurs in the common fowl a condition of disseminated lymphocytoma or lymphosarcoma, sometimes aleukemic, at other times associated with an increase of the white cells of the blood, this increase consisting in the appearance in great numbers of cells of the large lymphocyte type and apparently identical with the lymphoid cells of the tumor-nodules.

The aleukemic and leukemic conditions must, therefore, be regarded as genetically related, if not actually one and the same process, in different stages. The same thing is true of the leukemic and aleukemic lymphomata or lympho-sarcomata found in man and observed also in the dog; and occurring probably in all other animals in which leukemia has been discovered. The wide-spread occurrence of these affections may be taken as throwing some light upon the nature of the leukemic process. In this respect it resembles the malignant

tumors, these also showing a wide distribution throughout the lower animals. Such a wide distribution speaks against the existence of any specific parasite. In other respects, too, these conditions, both the leukemic and aleukemic, are comparable to malignant tumors. The formation of metastases, the infiltrative and destructive growth, the failure of inoculations and transplantations, etc., all favor the view that they are neoplasms, and present the same problems as do the malignant tumors.

If to one laboratory in the course of a few years there come four cases of aleukemic lymphocytoma and two of leukemic in the common fowl, its occurrence in this animal cannot be so very rare. The opportunity should, therefore, be taken, whenever possible, of making careful comparative pathological studies of these conditions with the view of throwing some light upon the problems of their occurrence in man. As interesting a field is opened here as in the case of carcinoma of the lower animals.

CONCLUSIONS.

1. Aleukemic and leukemic forms of lymphocytoma exist not infrequently in the common fowl, in all respects analogous to the similar conditions existing in man.

2. As in man, the two conditions appear to be genetically related, or different stages of the same process.

3. There is no evidence of an infective causal agent.

4. The essential nature of the two conditions would lead to their interpretation as malignant neoplasms. The problem of leukemia, then, becomes identical with that of malignant neoplasms in general.

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PLATE 14.

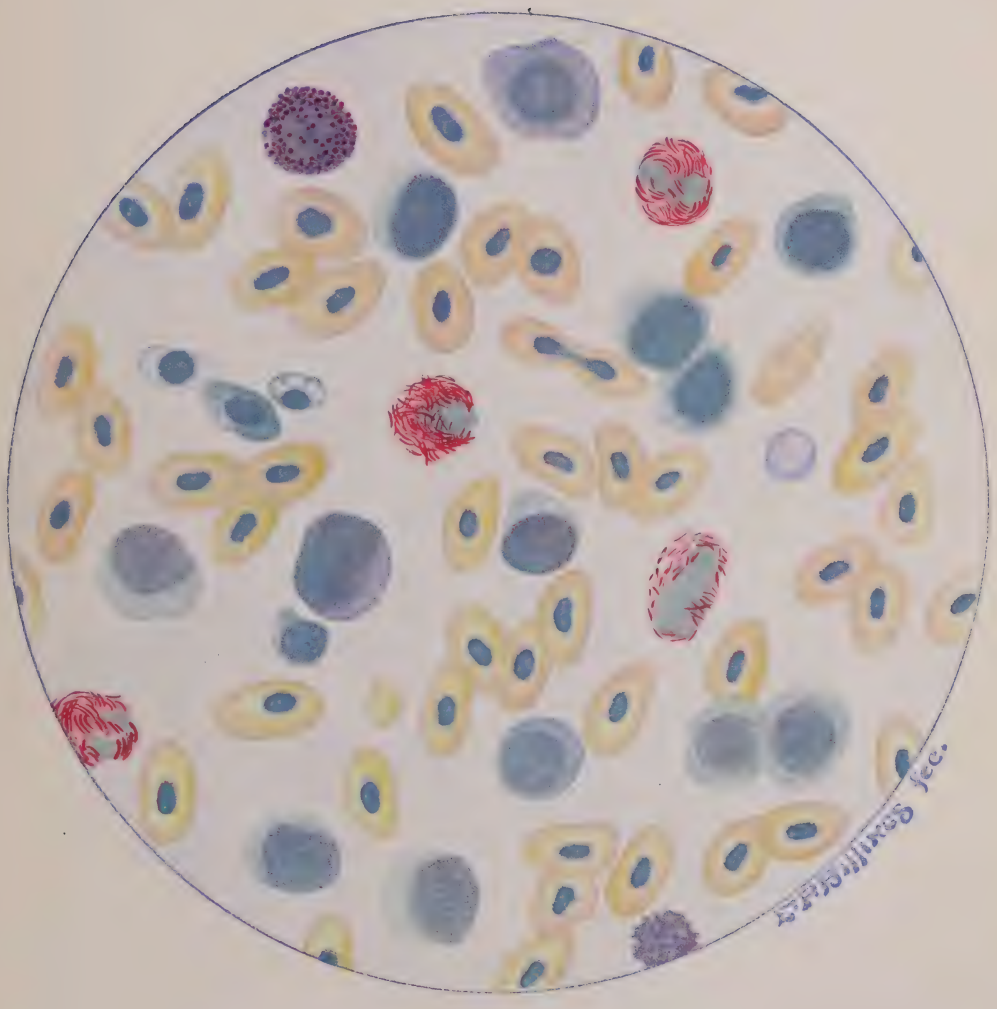

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PLATE 14.

Leukemia of the Fowl.

A METHOD FOR THE SIMULTANEOUS PASSAGE OF MANY PARAFFIN SECTIONS THROUGH THE MORE DIFFICULT STAINS.*

FRANCIS PEYTON ROUS.

(From the Pathological Laboratory, University of Michigan, Ann Arbor, Mich.)

NUMEROUS attempts have been made to find means whereby serial paraffin sections, or duplicate preparations intended for class-work, may be stained simultaneously; and, so far as regards stains that demand merely a transfer of the sections from one solution to another, these attempts have had success. Thus the Schmorl "celloidin-plate" method, by which the paraffin sections are incorporated in a film of celloidin and then manipulated, fulfils all the requirements for hematoxylin and eosin or other simple staining and counter-staining technic. When it is inadmissible—as, for instance, in clearing by turpentine, which affects celloidin—one may employ a glass or metal rack, such as that of Neumayer, to carry many slides on which the preparations have previously been fixed, or may, as recommended by Apathy, space the slides by means of bits of glass rod between each two, unite them all with rubber bands, and carry them through as one piece. But beyond the reach of these there remain a number of the processes for differential staining—Gram's stain, that of Weigert for fibrin, the amyloid and mucin stains, etc.—which, since they involve a careful differentiation of the dyes, blotting, and other detailed attention, have seemed to forbid the collective handling of many sections. To meet the conditions for simultaneous staining here involved some new method is needed. I have therefore devised the one that follows:

A clean glass plate is warmed over the burner and rubbed on one surface with a bit of paraffin of 36° C. melting-point, so that a thin coat of this, melted, is left. Cover-glasses to which the sections have been fixed in the usual manner are then dropped, with the free side down, on this coated surface, and at once adhere closely by reason of the film of paraffin. Since the paraffin about the sections themselves is of much higher melting-point than that between the cover-glasses

* Received for publication, February 12, 1907.

and plate, the procedure need not alter it. Any excess on the coated surface may be wiped off from one corner of the plate, which is then allowed to cool, and next placed in xylol. This removes all exposed paraffin, that of the sections with the rest, but cannot affect such as is between the plate and cover-glasses, by reason of their close apposition; and the plate is now ready, with the sections temporarily part of it, for the solutions necessary to the stain. Though no special instrument is required wherewith to handle the plate, a wire "holder" like that figured will be found convenient. Differentiation and blotting are controlled as for a single section, with results as precise as though attention had been given to but one.

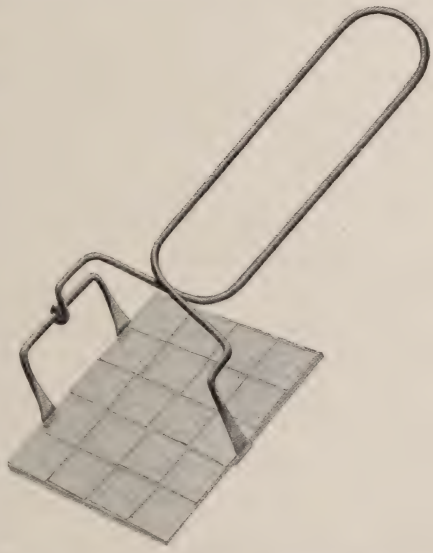


FIG. 1.—Plate in wire "holder" bearing 20 cover-slips with paraffin sections. The cover-slips are attached to the plate by means of paraffin melting at 36°C., and are thus ready to be passed as one through the staining solutions.

To loosen the cover-slips from the plate for mounting it is only needful that the last medium before the balsam be at the body temperature. With the paraffin thus melted, the cover-slips become individuals once more, and may be picked off, blotted, and mounted. As xylol or another medium dissolving paraffin is ordinarily the last one through which sections are "run up" into balsam, the film on the reverse of the cover-slips is dissolved off as soon as they are freed from the plate; but should such a medium not be used, it will be found necessary to clean this film from the cover slips when they have been mounted.

In such way it is feasible, with trays, to stain on one 4×5 glass plate 20 paraffin sections fixed to separate cover-slips of ordinary size; or, if jars are employed, 40 such sections, since both sides of the plate may be utilized. The Gram stain and other stains "in the cold" for bacteria, the Weigert fibrin stain, muchaematin for mucin, the cresylecht violet for mucin and amyloid,

and Weigert's elastic tissue stain lend themselves well to the procedure.

The limitations of the method are obvious. The newly coated plate with cover-slips applied cannot be left more than five or ten minutes in xylol; else the paraffin will slowly dissolve from under the margins of the slips, and the spaces so afforded will later prove a drawback in that they carry portions of one reagent into the next. More important, should the final fluid before the balsam be such as will take out the stain rapidly, the last section of a batch, mounted from this fluid, will differ somewhat in color from the first, owing to its longer sojourn therein. In this case the medium just preceding may be warmed to 36°, and the cover-glasses, thus loosened, passed on separately. The possible alteration in stain caused by a short stay of the preparations in a fluid heated to the body temperature may be cited as an objection, but in practice this difficulty is inconsiderable. During summer heat paraffin melting at 36° C. may not prove sufficiently stable to use in attaching cover-slips to the plate, and one of slightly higher melting-point may be adopted instead.

The method is, of course, valid for the routine stains of a laboratory, but seems, as already mentioned, to have a special field in those procedures more difficult technically.

AN ATTEMPT TO PRODUCE AN ATYPICAL EPITHELIAL GROWTH BY INJECTION OF SCHARLACH R. IN OLIVE OIL; A CONTROL OF FISCHER'S EXPERIMENTS.*

CLARENCE SNOW.

(From the Pathological Laboratory, University of Michigan, Ann Arbor, Mich.)

PERHAPS no work bearing on the etiology of carcinoma has been more suggestive and has caused more comment than that recently reported from Ribbert's laboratory by Dr. B. Fischer.[1] On account of the important bearing his results may have on our ideas of the origin of these malignant growths it seemed desirable that the work be repeated by other observers. Accordingly, at the suggestion of Dr. Warthin, I have attempted to do this.

After having reviewed the previous attempts to produce a destructive atypical epithelial proliferation by means of irritation, and concluding that no progress was to be made in that direction, Fischer was led by consideration of the work of Loeb on parthenogenesis to ask if he could not, by producing a proper physico-chemical environment, induce an epithelial proliferation entirely analogous to that produced by Loeb in the unfertilized egg of the sea-urchin. Ribbert had previously pointed out that in carcinomatous growths arising from the skin there is often an inflammatory infiltration of the subepithelial connective tissue, and Fischer therefore thought to bring about his desired condition by producing a chronic inflammation of the papillary bodies. He accordingly implanted bits of sterile muscle, liver, spleen, and other tissues beneath the epithelium of rabbits' ears. The only result was a slight thickening of the epithelium when the inflammatory reaction reached the germ layer. He then tried injecting agar-agar, with and without calcium salts, and in one case found that the epithelium did proliferate, and grew down around the agar masses. Further experiments upon other animals and upon the same animal failed to give like results, and he concluded that in the

* Received for publication May 1, 1907.

[1] *Münch. med. Wchnschr.*, 1906, 53, p. 2041.

one apparently successful case some epithelial cells had been carried in with the injecting needle, and in their new location had proliferated. Next he used olive oil, on account of the slowness with which it is absorbed, the slight chronic inflammation which it produces, and the ease with which it can be made to permeate the tissues. The pure oil caused, after several weeks, a thickening of the epithelium, and in some cases the development of short processes, but no atypical growth. Various substances were added to the oil without further result until the fat-staining dyes Scharlach R. Sudan III, and Indo-phenol were used.

According to Fischer, when a saturated solution of Scharlach R., in pure olive oil, which he calls "scarlet oil," was caused to permeate the tissues beneath the epithelium a marked change followed. The tissues became hyperemic, infiltrated with round cells, connective-tissue giant cells were formed, and there developed a myxomatous-like connective tissue. He further states that within a few days the superficial epithelium, the cells of the rete Malpighii, and those of the hair follicles and gland ducts showed mitoses, typical and atypical; that processes of epithelium grew down into the subjacent tissue toward the oil droplets, and in many cases came to surround them. The picture so produced was, in his opinion, to be distinguished in no way histologically from a squamous-celled carcinoma in man. He says that in some cases the cartilage was invaded, and that in one case he found an epithelial plug growing in a lymph channel.

He thought the growth was independent of the inflammatory reaction since the processes penetrated beneath the inflammatory zone, and that it was not the result of irritation since neither olive oil alone nor other irritants produced the same effect, and since no such result followed repeated painting of the rabbit's ear with scarlet oil through a period of several months.

So long as the tissues contained scarlet oil the growth continued, but when this was exhausted growth ceased and the epithelial plugs underwent cornification, resulting in cholesteatomatous masses which broke and discharged outward.

He states that he obtained the same though less marked results by the use of dry Scharlach R.

In general, within three weeks after injection, visible growth could

be seen over the area of injection. Drawings are shown of tissue excised after seven to 24 days, one injection having been made in some cases, two in others.

Fischer sees in these results the evidence of a chemotactic influence of the scarlet oil on the epithelial cells, and suggests that certain chemical groups, which he calls "attraxines," exist in the scarlet oil, and that these attraxines, which are specific in their influence, may under certain conditions develop in the body and give rise to carcinomatous proliferation of the epithelium. He promises further reports, and expresses the hope that in the meantime other workers will confirm his observations.

If these observations can be confirmed and the attraxine theory placed on a firm basis, it would without doubt go far toward explaining the etiology of carcinoma, and probably of other malignant tumors. It therefore seemed important that control experiments be done, and accordingly immediately after the appearance of Fischer's paper, work to that end was begun in this laboratory.

Unfortunately Fischer gives no detailed account of his technic, so that in repeating the experiments I could follow only the bare outlines of his methods.

The work done here is as follows: Six white rabbits were used, three about two months old and three full grown. A saturated solution of Scharlach R. in olive oil was made up, the Scharlach R. used having been obtained in Berlin from E. Leitz, the oil being Lucca crème.

This was sterilized by heating it repeatedly, care being taken that it was not heated enough to cause any alteration in color. This was injected under the skin on the outside of the ears of the rabbits, the fluid being forced in under slight pressure until the tissues were permeated for some distance around the needle. At various times bits of the permeated tissues were excised and immediately fixed in mercuric chloride, imbedded in paraffin, and sectioned. In all cases the tissues still contained Scharlach R., and in most cases oil was still present. A summary of the results follows.

Experiment 1.—Young rabbit No. 1. Seven days after injection. The ear about the point of injection was swollen and congested. Section showed spaces in the connective tissue made by oil droplets; many hair follicles cut at various depths, some

extending almost to the cartilage, and many sebaceous glands. There was no evidence of proliferation in any of the epithelial structures, neither typical nor atypical mitoses, nor any evidence of an inflammatory reaction on the part of the connective tissue.

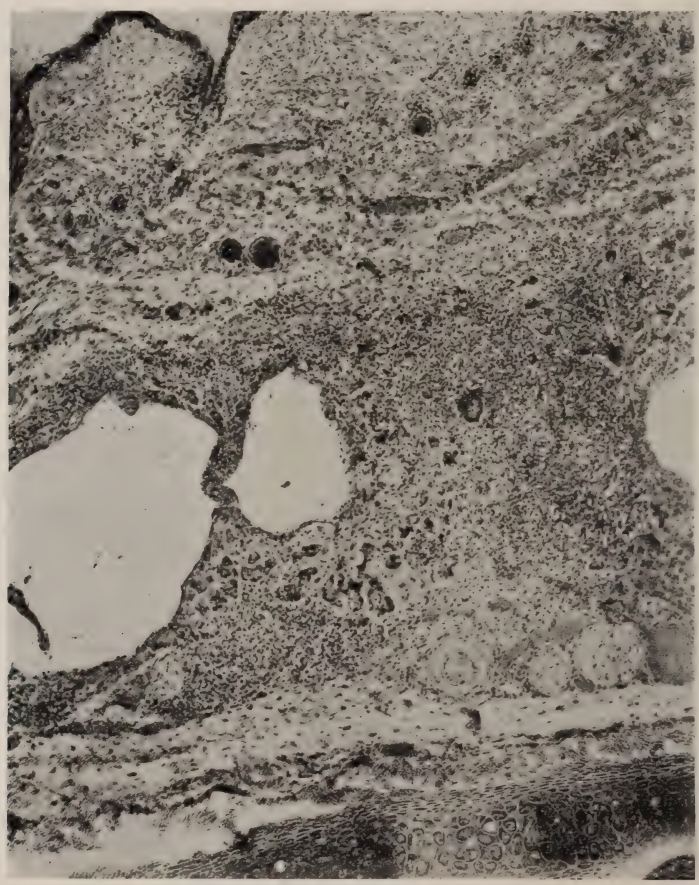


FIG. 1.—Section from ear of young rabbit 28 days after injection with scarlet oil. Note the marked inflammatory reaction, the large plasmodial masses surrounding the open spaces, the outlines of the giant cells not being made out. There is no epithelial proliferation.

Experiment 2.—Young rabbit No. 2. Twelve days after injection. The ear about the area of injection was very slightly, if at all, swollen, and somewhat congested. Section showed congestion, edema and slight mononuclear infiltration of the connective tissue. There was no evidence of epithelial proliferation, no mitoses, typical or atypical.

Experiment 3.—Old rabbit No. 4. Seventeen days after injection. The ear showed no pathological changes except redness from the presence of the dye. Section

showed large open spaces in the connective tissue which had been occupied by oil droplets, the connective tissue was edematous and showed slight mononuclear infiltration. There was no evidence of epithelial proliferation, neither typical nor atypical mitoses.

FIG. 2. — Same as Fig. 1. Higher magnification.

Experiment 4.—Small rabbit No. 2. Nineteen days after injection. The ear showed no pathological changes other than redness due to the dye. Section showed collapsed spaces made by oil droplets, about which there was hyperplastic connective tissue infiltrated with round cells, mainly plasma cells, and many multinucleated giant cells of the type of foreign-body giant cells. There was no evidence of proliferation on the part of the epithelium, neither typical nor atypical mitoses.

Experiment 5.—Old rabbit No. 4. Twenty-four days after injection. The ear showed only redness due to the dye. On section the connective tissue was very ede-

matous with slight mononuclear infiltration. There was no sign of epithelial proliferation, neither typical nor atypical mitoses.

Experiment 6.—Young rabbit No. 2. Twenty-eight days after injection. The ear showed only redness due to the presence of the dye. Section showed congestion,

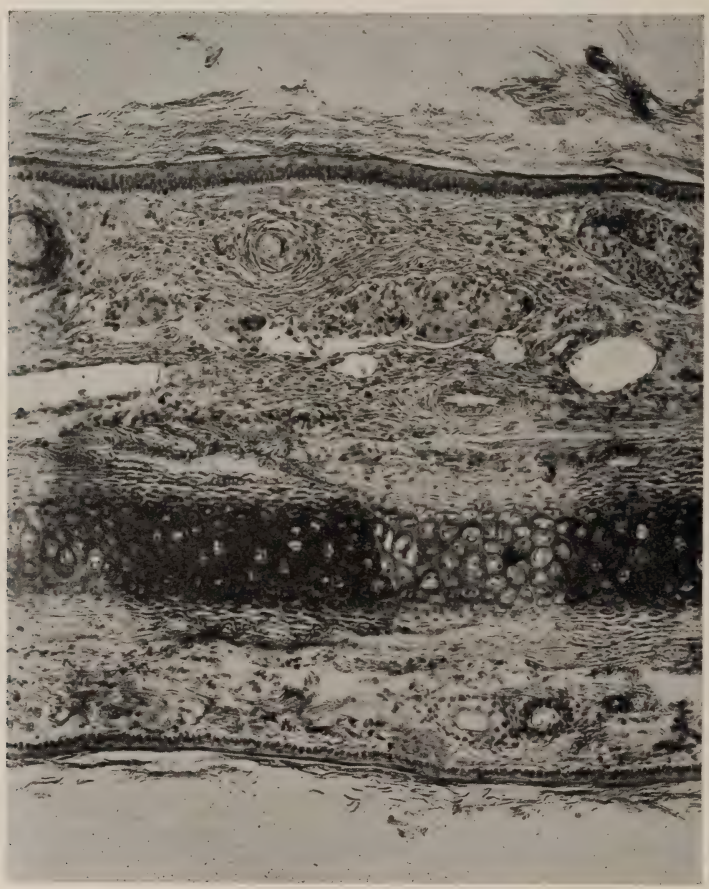


FIG. 3. — Section from ear of young rabbit 32 days after injection of scarlet oil, showing open spaces made by oil droplets, bordered by large plasmodial masses. Note slight inflammatory reaction and absence of epithelial proliferation.

hyperplasia of connective tissue, marked mononuclear infiltration and the presence of numerous open spaces made by oil droplets. Around these spaces there was a very marked infiltration of plasma cells and great numbers of giant cells of the character of foreign-body giant cells. In some places the walls of the open cavities consisted wholly of plasmodial masses of giant cells, the cell outlines not being visible. One of these giant cells showed a cell inclusion, the only evidence of phagocytosis seen in them.

The blood vessels about the spaces were congested. The dermis over them was thinned, but there was not the slightest evidence of proliferation on the part of the epithelial elements, either of the epidermis or of the hair follicles or of the glands. The only pathological change in the dermis was its slight thinning over the zone of reaction about

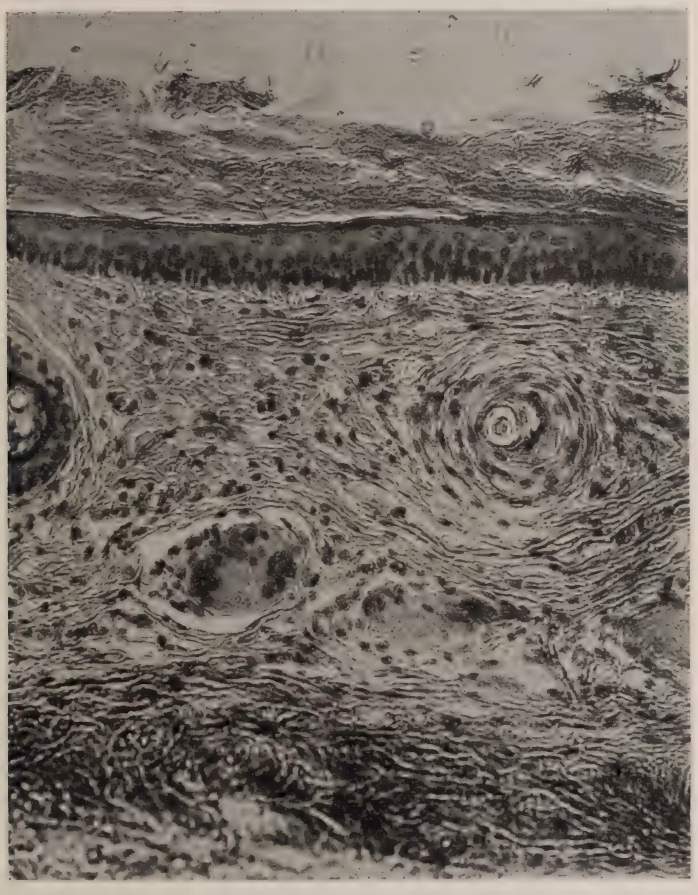


FIG. 4. — Same as Fig. 3. Higher power.

the spaces and an apparent increase in the number of wandering cells. There were no mitoses ,either typical or atypical, in the epithelium and no signs of epithelial ingrowth.

Experiment 7.—Large rabbit No. 5. Thirty days after injection. The ear was red, due to the presence of dye. The sections showed open spaces surrounded by hyperplastic connective tissue, slight mononuclear infiltration, but the reaction about the spaces was very slight indeed when compared with that of the younger rabbit just

described. There was no evidence of epithelial proliferation. No mitoses, typical or atypical.

Experiment 8.—Small rabbit No. 3. Thirty-two days after injection. The ear showed only redness due to the presence of dye. The sections showed smaller

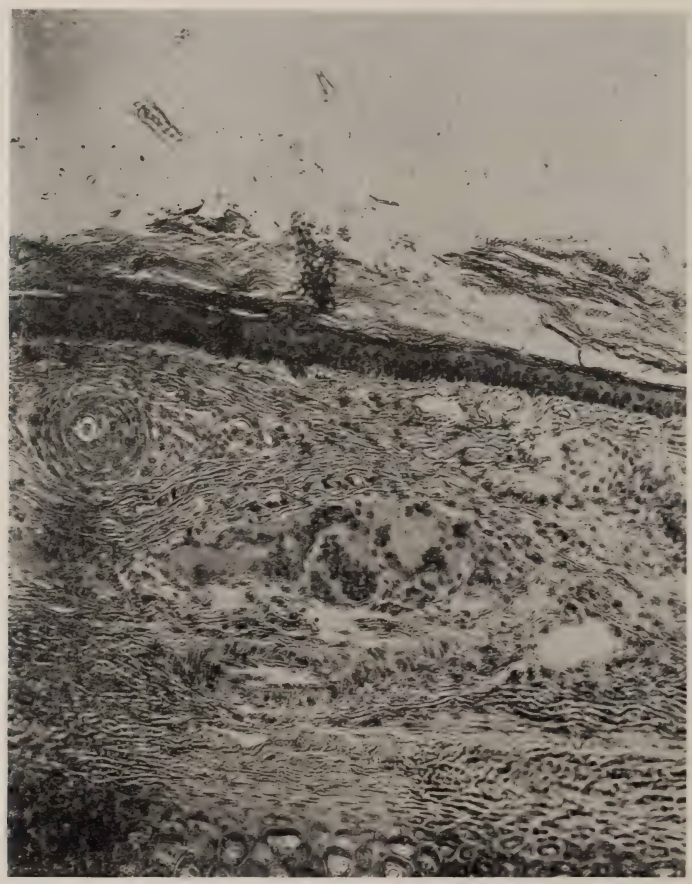


FIG. 5. — Same as Fig. 3. Higher magnification.

spaces than the previous sections, most of the oil having been absorbed, leaving behind the dye. About these smaller spaces the connective tissue was hyperplastic and infiltrated with plasma cells, the walls of the spaces being largely made up of very large multinucleated plasmodial masses of the character of foreign-body giant cells. There was no sign of epithelial proliferation, neither typical nor atypical mitoses.

Experiment 9.—Large rabbit No. 5. Forty-four days after injection. The ear showed only slight redness due to the dye. The sections showed large open spaces about which there was slight reaction, the connective tissue about them being hyperplas-

tic and slightly infiltrated with plasma cells. The spaces were lined by thin flattened endothelium. There was no evidence of epithelial proliferation.

Experiment 10.—Large rabbit No. 6. Sixty-one days after injection. The ear was slightly red, due to the presence of dye. It bore, though not near the site of injection,

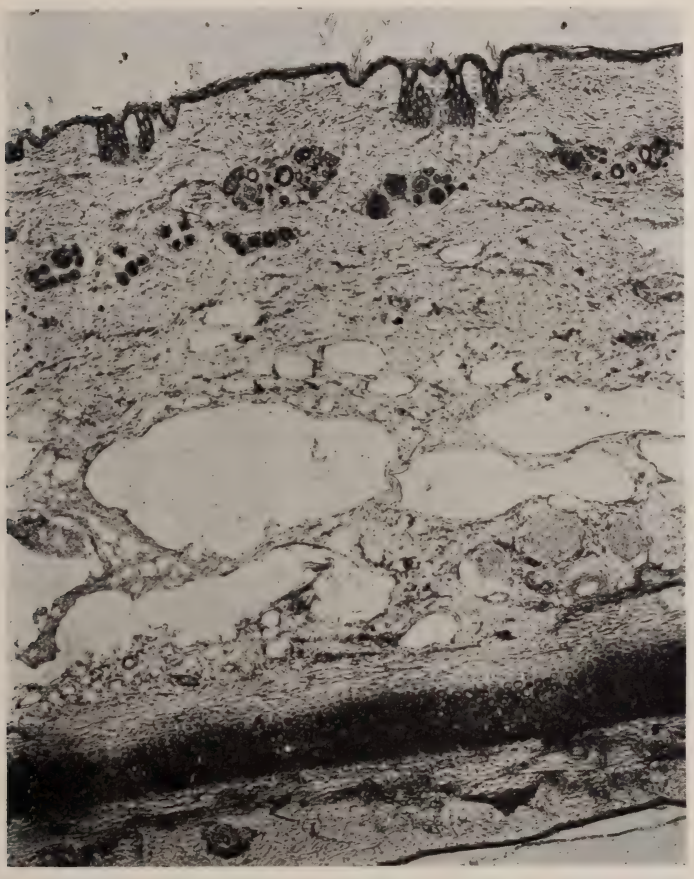


FIG. 6. — Section from ear of large rabbit 44 days after injection of scarlet oil, showing open spaces made by oil droplets. Note absence of inflammatory reaction and of epithelial proliferation.

a small indurated area. On section this proved to be a small sebaceous cyst. About this there was a slight inflammatory reaction, but the balance of the tissue showed no change aside from a hyperplasia of connective tissue. There was no evidence of epithelial proliferation, neither typical nor atypical mitoses.

These results show that the scarlet oil had no influence whatever on the epithelial cells, although it was present in the tissues at times

varying from seven to 61 days. It acted simply as a slight irritant, inducting a low-grade chronic inflammation. It is interesting to note that giant cells were formed as a result of the irritation only in the case of the young rabbits.

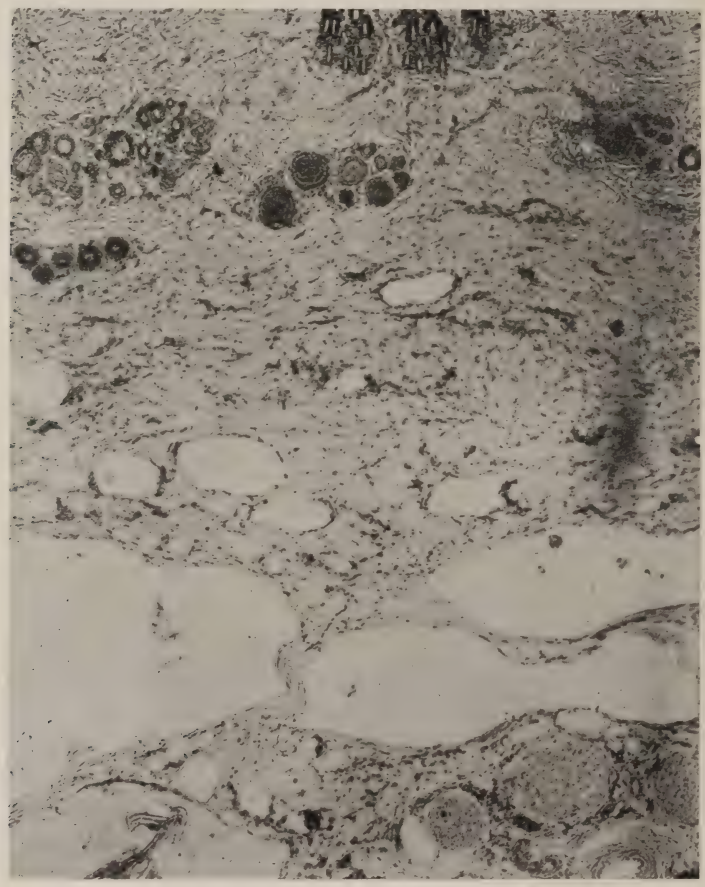


FIG. 7. — Same as Fig. 6. Higher power magnification.

So far as we can tell the work of Dr. Fischer has been duplicated. The character of injection and the time elements at least are identical, so far as we are able to tell from his paper, and yet our results are entirely negative. The question naturally arises, then, if Fischer's findings allow of any other explanation. Is it possible that he mistook foreign-body cells with inclusions for epithelial plugs?

As he does not mention the age or variety of the rabbits used we are in doubt as to whether this will prove to be the explanation. If he used only young rabbits such error might be possible, but this would

FIG. 8. — Section of rabbit's ear, normal, showing depth to which hairs penetrate. If the follicles were slightly hyperplastic, the picture might easily be mistaken for a squamous celled carcinoma.

not apply to old rabbits, for, as just pointed out, we found that foreign-body giant cells were not developed by the old animals.

There seems to remain only one other possibility, which we mention with hesitation, that physiological appearances were mistaken for pathological. Sections taken from different parts of the ear show great variation in the number of hairs and sebaceous glands and the depth to which these penetrate toward the cartilage. There would also be great variation between rabbits with smooth coats and those with rough coats. Larger hairs run obliquely at a slight angle for a long distance beneath the epithelium in some parts of the ear.

Oblique sections and even those at right angles will show in some areas normally a dermis set full of epithelial structures cut at various angles, longitudinally and transversely. In some cases the epithelia structures reach nearly, if not quite, to the cartilage. Fischer's Fig. 4 could easily be explained by such a section, as also his Figs. 1, 2, and 5. In Fig. 3 the upper part of the section could be normal structures cut obliquely, while the epithelium around the oil spaces might have been the foreign-body cells. Since Fischer's plates are made from drawings rather than from photographs, an absolute judgment is impossible.

We hesitate at offering such an explanation, and certainly do not wish to accuse Dr. Fischer of making such a gross error of judgment, but the difference in our results certainly calls for some explanation.

Further control work should be done along this line, inasmuch as my experiments seem to leave the attraxine theory on rather a weak foundation.

Dr. Warthin has controlled my work throughout and confirmed my observations. To him I am greatly indebted for this and for the great interest he has shown in the progress of the work.

NOTE.—After finishing the above work a similar set of experiments with Sudan III, Indo-phenol, and other similar substances was undertaken as a further control of Fischer's work. In the ear of a Belgian hare examined 12 days after injection of Sudan III in olive oil, a well-marked hyperplasia of the epithelium of the hair follicles over the area of injection was seen. Serial sections showed no atypical proliferation, the epithelium of the follicles showing only a simple hyperplasia. In the ear of a young, white rabbit injected at the same time with Sudan III in olive oil no hyperplasia was seen. This suggests the possibility that different varieties or species may react differently. Further work along these lines is under way and will be reported later.

NOTES ON THE DETERMINATION OF *B. COLI* IN WATER.*

FRANCIS F. LONGLEY AND WARREN U. C. BATON.

(From the Laboratories of the Filtration Plant, Washington, D. C.)

THE best laboratory evidence of the sanitary quality of a water supply is to be obtained from a study of the bacterial flora of the water, with special reference to organisms which may have had their origin in sewage.

The most typical of these sewage organisms, and the one most frequently looked for as an index of sewage pollution, is the colon bacillus.

The number of these organisms in a water supply has a considerable range, from a few hundred per c.c. in a grossly polluted water, down to practically none in some springs and deep wells, surface water under occasional conditions, and the effluent of a filter of high efficiency. When they are frequently present, the determination of the number of acid-forming colonies developing upon the litmus lactose agar plate of Wurtz gives an indication sufficient for all practical purposes of the polluted condition of the water. When they are quite absent from a water it is a simple matter to demonstrate the fact with reasonable certainty. The complications and the refinements in the determination of the presence of this organism are brought out in a long-continued routine examination of waters which lie between the two limiting classes, that is, in which the most delicate tests show *B. coli* to be sometimes absent and sometimes present, but in numbers too small for direct determination. Especially is refinement in this work necessary in determining the sanitary efficiency of a filter, under conditions which may bring forth critical inspection of the results obtained.

In the work of this laboratory we have accumulated a mass of data obtained in carefully conducted coli work, and we present it here in statistical form, with certain observations upon the various factors of the work, for the benefit of others engaged in similar studies.

* Received for publication March 25, 1907.

The work has been done entirely along the lines laid down in the *Report of the Standard Committee on Water Analysis of the American Public Health Association*, supplemented by various other means for gaining as broad a knowledge as possible of the work in hand.

In order to permit a more general interpretation of certain parts of the work presented herein, we have obtained data on gas formation on several different classes of waters, including surface waters in other localities and under other conditions of pollution, lake waters, and well waters. These tend, without exception, to substantiate our conclusions regarding the points in question.

For certain of these data, and for suggestions and criticisms in the preparation of this paper, we take this occasion to express our appreciation and thanks to Mr. Geo. C. Whipple, New York; and Mr. Karl F. Kellerman, of the Bureau of Plant Industry, Department of Agriculture, Washington, D. C.

OUTLINE OF WORK.

The discussion of the several parts of this work is taken up according to the following outline—

(1) GAS FORMATION.
   (*a*) Quantity of gas produced by *B. coli*.
   (*b*) Quantity of CO_2 produced by *B. coli*.
   (*c*) Diagnostic value of the fermentation test.
(2) LENGTH OF PERIOD FOR PRELIMINARY FERMENTATION.
(3) VALUATION OF SUBCULTURE TESTS.
(4) THE PRESUMPTIVE GAS TEST, AND ITS APPLICATION TO THE ROUTINE EXAMINATION OF POTOMAC WATER, IN COMPARISON WITH A COMPLETE CONFIRMATORY TEST.
(5) OTHER PRESUMPTIVE TESTS.
   (*a*) Wurtz litmus lactose agar plate.
   (*b*) Neutral red reaction.
   (*c*) Use of the bile salts.

(1) GAS FORMATION.

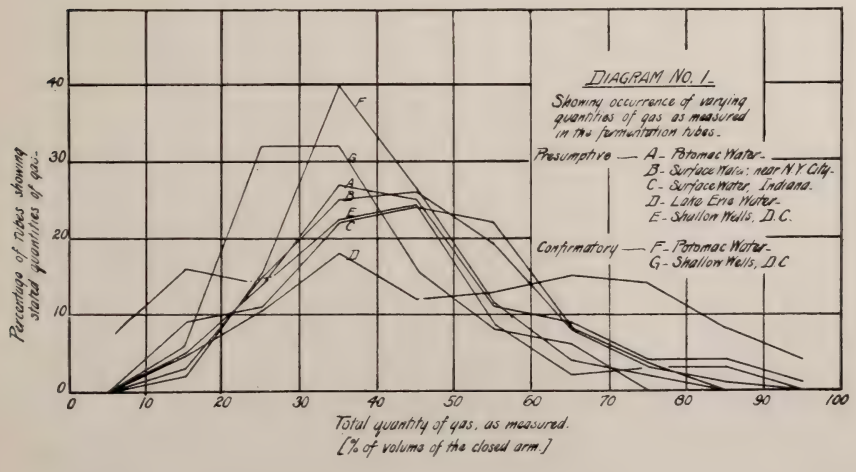
Of all the cultural reactions for the identification of *B. coli*, one of the most important is the formation of gas in dextrose broth. It has been generally taught that if the total amount of gas measured

in the fermentation tube is trom 25 to 70 per cent of the capacity of the closed arm; and of this gas, approximately two-thirds is hydrogen and one-third carbon dioxide, the test is considered typical. If the amount of gas is greater than 70 per cent or less than 25 per cent, but not less than 10 per cent, or if there is an amount of CO_2 greater than 40 per cent of the total gas, the test is considered atypical. If the quantity of gas is less than 10 per cent the test is negative.

This statement of quantities of gas and CO_2 is applied most specifically to the presumptive gas test for *B. coli.*

Authorities have apparently avoided stating any definite limits for gas production and CO_2 in the subculture fermentation tube which forms a part of the confirmatory test, dismissing the subject with the very general statement that *B. coli* ferments certain carbohydrate broths with the formation of about 50 per cent of gas, of which about one-third is CO_2. There is good reason for the omission of a definite statement on this point.

The quantities of gas and CO_2 are often very erratic, even in pure culture, though a process of rejuvenation will often restore the typical gas formation. The fermentation of sugar broths is the only one of the subculture tests which is of a quantitative nature, and it is unfortunate that more significance can not be attached to this. Our experience, however, does not bring us any nearer to this end.



Consideration of this point will be taken up under two heads, namely: (*a*) Quantity of gas; (*b*) Quantity of CO_2.

(*a*) *Quantity of gas.*—We have tabulated the gas formation, classified according to the percentage of total gas measured, in routine coli work on the Potomac water, and also on other classes of water. The gas formation in the preliminary or presumptive tubes, and in the subculture or confirmatory tubes, are tabulated separately. Diagram 1 shows this distribution more graphically. These tables and curves are quite typical and need no comment, except to point out the greater maximum and the narrower limits in the case of the confirmatory gas formation.

TABLE 1.

(*a*) PERCENTAGE OF PRESUMPTIVE FERMENTATION TUBES SHOWING THE STATED QUANTITIES OF GAS.

Class of water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Potomac water	452	0.2	5	14	27	25	11	9	4	4	1
Surface water near New York City	552	0	3	15	25	26	19	8	3	1	0
Surface water—Indiana	96	0	9	11	22	24	22	8	4	0	0
Lake Erie water	175	0	5	11	18	12	13	15	14	8	4
Shallow wells, Washington, D. C.	98	7	16	14	22	24	9	2	3	3	0

(*b*) PERCENTAGE OF CONFIRMATORY FERMENTATION TUBES SHOWING THE STATED QUANTITIES OF GAS.

Potomac water	528	0	2	15	40	26	11	4	2	0	0
Shallow wells, Washington, D. C.	50	0	6	32	32	16	8	6	0	0	0

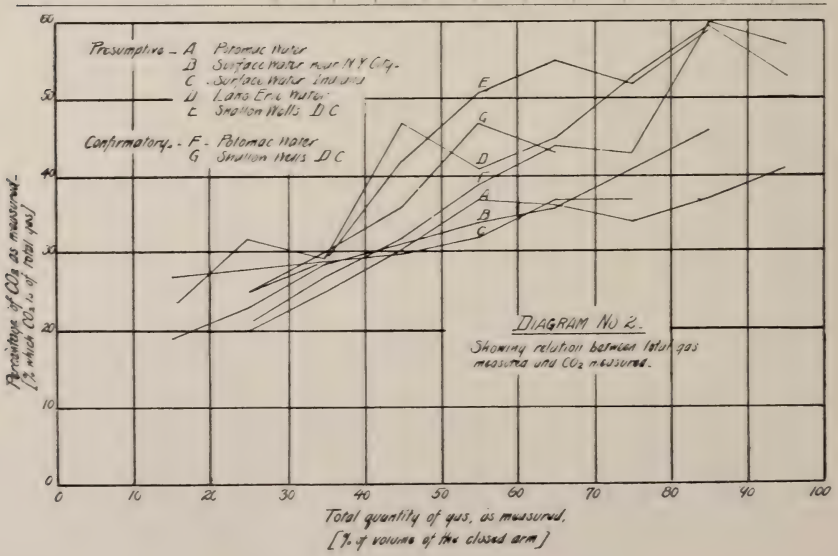


TABLE 2.

(*a*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN PRESUMPTIVE FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Class of Water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Potomac water	452	..	..	20	25	31	37	36	34	37	41
Surface water, near New York City	552	..	19	23	29	31	34	36	41	46	..
Surface water, Indiana	96	..	27	28	29	30	32	37	37	..	..
Lake Erie water	175	..	23	32	29	47	41	45	53	60	53
Shallow wells, Washington, D. C.	98	..	..	25	29	42	51	55	52	59	..

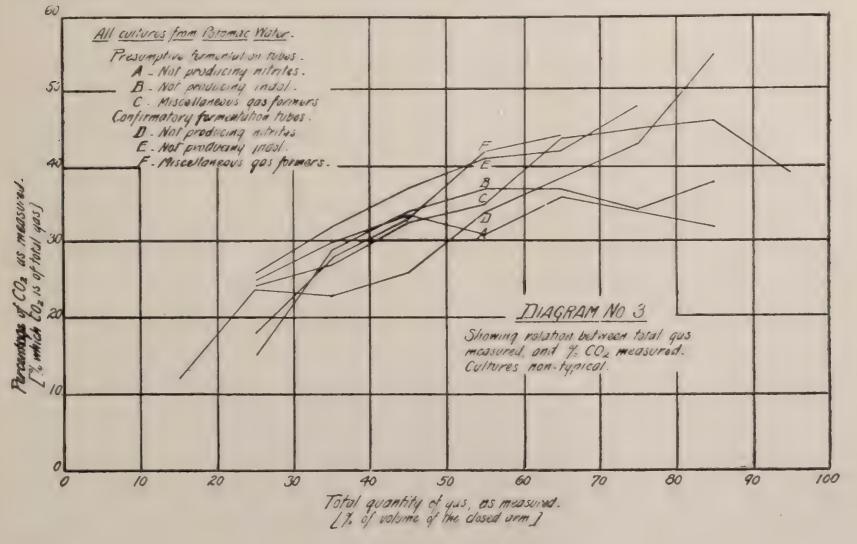
(*b*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN CONFIRMATORY FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Potomac water	528	..	..	21	27	32	39	44	43	60	57
Shallow wells, Washington, D. C.	50	..	..	25	30	36	47	43	..	..	..

TABLE 3.

(*a*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN PRESUMPTIVE FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS

Cultures from Potomac Water	No. of Observations	Total Quantity of Gas Between									
		0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Not producing nitrites	39	..	..	25	30	34	31	36	..	32	..
Not producing indol	97	..	..	15	29	34	37	37	34	38	..
Miscellaneous gas formers	69	..	..	24	27	33	35	44	45	46	39



(*b*) AVERAGE PERCENTAGE OF CO_2 PRESENT IN CONFIRMATORY FERMENTATION TUBES SHOWING STATED QUANTITIES OF GAS.

Not producing nitrites.....	40	..	12	24	23	26	34	..	43	55	..
Not producing indol.....	106	..	..	26	32	37	41	42	48	..	..
Miscellaneous gas formers.....	76	..	..	18	28	33	42	44	..	..	..

(*b*) *Quantity of CO_2.*—It has been generally stated that the quantity of CO_2 from a typical culture of coli, as determined by absorption by sodium hydrate, should be about one-third of the total volume of gas produced. This of course has been looked upon as only an approximate relation, and some latitude has been permitted in its application. We have long noticed, however, that this relation did not hold, but that there was instead a more or less regular variation in percentage of CO_2 depending on the total quantity of gas present.

We have therefore made a statistical analysis of our accumulated data with special reference to this relation between gas and CO_2, and in the same tables we give similar figures for the other classes of waters.

In Diagram 2 these relations are shown graphically to better advantage. It will be seen that there is an unmistakable increase in the percentage of CO_2 measured, with an increase in the total quantity of gas. Some of the curves are quite irregular, and there seems to be a tendency for the curve to fall off as the total quantity of gas approaches 100 per cent. This does not interfere with the general conclusion, however.

The relation we have pointed out does not seem to have any diagnostic value. As will be explained later we think it is a purely physical phenomenon, which perhaps obscures, rather than renders plainer, the significance of a quantitative gas test. The increase of CO_2 with increased total gas is not peculiar to *B. coli*, but seems to be true generally of gas-forming organisms. This is evident from the figures given in Table 3, and from Diagram 3, which is merely an extension of the analysis of our data to include the atypical forms of *B. coli* that fail to produce nitrites and indol, and a miscellaneous lot of gas-producing cultures that are entirely without the coli classification.

No theory has ever come to our attention to explain the variable

relations between gas and CO_2; nor have we been able to suggest any theory that would account for the *production* of varying proportions of the constitutent gases, by organisms of one kind, under quite constant conditions. It has been suggested as a much more probable theory that the gas as *produced* might have a more or less constant composition; and that the explanation for the variations we have observed might be found in some physical phenomena connected with the incubation of the cultures.

The gas produced in the fermentation tube by *B. coli* and other organisms of that general type probably consists of CO_2, hydrogen, nitrogen, methane, and perhaps other light hydro-carbons. CO_2 is readily absorbed in water, while hydrogen and other light gases are scarcely absorbed at all, the quantity being less than 5 per cent of the quantity of CO_2 that could be absorbed at the incubator temperature of 40° C., other conditions being equal. Therefore with the small quantities of gas to be measured, and the rather crude methods of measurement in routine work, the absorption of gases other than CO_2 may be neglected.

Under the conditions of incubation, the temperature and pressure of the gases in the closed arm of the fermentation tube and also the area of liquid exposed to absorption are substantially constant. Then leaving out of consideration other refinements of this problem, the actual quantities of CO_2 absorbed in different tubes will probably be about equal. The length of time the gases are exposed for absorption, from the time they begin to form until they are measured, may have an influence upon the quantity absorbed, and may account for some of the observed irregularities; but in general, it is probable that about equal quantities of CO_2 will be retained in solution in different fermentation tubes.

If this be so we will have the following conditions:

(*a*) A small quantity of total gas of approximately constant composition as produced; an approximately constant quantity of CO_2 absorbed, or held in solution as fast as formed depending upon temperature, pressure, etc.; and consequently a relatively small quantity or percentage of CO_2 remaining to be measured by absorption with sodium hydrate.

(*b*) A large quantity of total gas of same composition as above;

same constant quantity of CO_2 absorbed or held in solution; and consequently a relatively large quantity or percentage of CO_2 remaining to be measured by absorption with sodium hydrate.

And similarly for other quantities of total gas within or beyond the range of the cases here given; so that in accordance with this theory we should expect to find CO_2 present above the liquid in the fermentation tubes in percentage quantities varying with the total quantities of gas present.

This can be put in mathematical form. Let "a" be the approximately constant quantity of CO_2 absorbed under the given conditions of temperature and pressure expressed as the percentage of the total volume of the closed arm.

Assume that the percentage of CO_2 actually produced is 75 per cent, which is approximately the figure we have found under the most favorable conditions, as will be shown further on.

Then we may make a table as follows:

TABLE 4.

Total Quantity of Gas		Residual Gases =25 Per Cent	Percentage of CO_2 as Measured	When a=		
Measured	Produced			40%	50%	60%
10	$10+a$	$0.25\,(10+a)$	$\frac{10-0.25\,(10+a)}{10}$	..	..	..
20	$20+a$	$0.25\,(20+a)$	$\frac{20-0.25\,(20+a)}{20}$	25	$12\frac{1}{2}$	..
30	$30+a$	$0.25\,(30+a)$	$\frac{30-0.25\,(30+a)}{30}$	42	33	25
40	$40+a$	$0.25\,(40+a)$	$\frac{40-0.25\,(40+a)}{40}$	50	44	37
50	$50+a$	$0.25.(50+a)$	$\frac{50-0.25\,(50+a)}{50}$	55	50	45
60	$60+a$	$0.25\,(60+a)$	$\frac{60-0.25\,(60+a)}{60}$	58	54	50
70	$70+a$	$0.25\,(70+a)$	$\frac{70-0.25\,(70+a)}{70}$	61	57	53
80	$80+a$	$0.25\,(80+a)$	$\frac{80-0.25\,(80+a)}{80}$	63	59	56
90	$90+a$	$0.25\,(90+a)$	$\frac{90-0.25\,(90+a)}{90}$	64	61	58
100	$100+a$	$0.25\,(100+a)$	$\frac{100-0.25\,(100+a)}{100}$	65	63	60

This table is shown diagramatically, and the relations between total quantity of gas measured, and total percentage of CO_2 measured are entirely in accordance with the facts, if the above assumptions

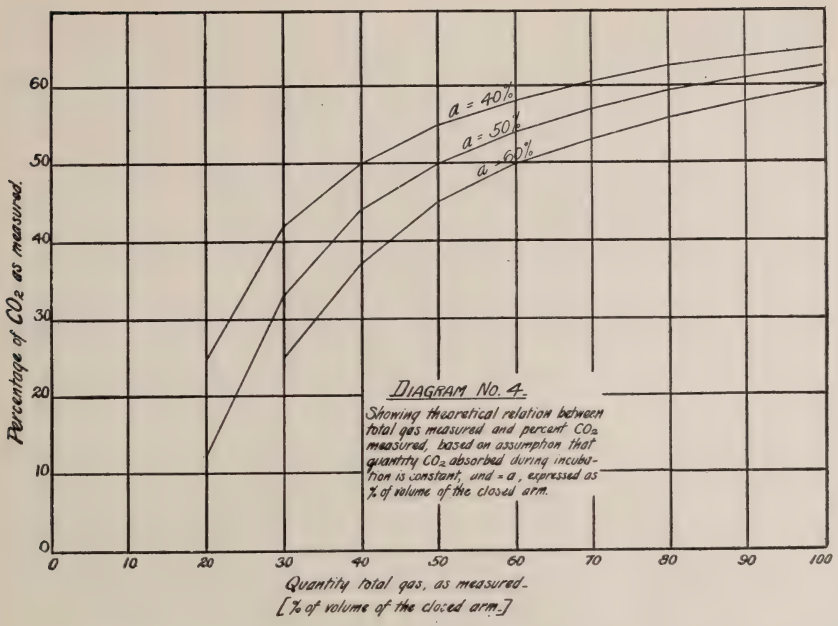


DIAGRAM NO. 4.

Showing theoretical relation between total gas measured and percent CO_2 measured, based on assumption that quantity CO_2 absorbed during incubation is constant, and = a, expressed as % of volume of the closed arm.

are not unreasonable. This is true, not only for the cultures of typical *B. coli*, as determined by complete confirmatory tests, but also, with rather more irregularities, for the atypical or paracolon forms, for the miscellaneous gas formers, and for the presumptive tests on the other classes of water. This would seem to indicate that for a great many of the gas-forming organisms ordinarily found in water supplies, the composition of the gas so formed was more or less constant.

In accordance with this theory we should expect to find, too, some constant relation between total gas and CO_2 if the tubes were incubated under conditions that would prevent the absorption by the media of any of the CO_2 as formed. Efforts were made to produce these conditions.

The closed arms of the fermentation tubes were filled with CO_2 in such a manner as to preserve their sterile condition, after which the tubes were placed under pressure in an atmosphere of CO_2 for a time. The CO_2 remaining unabsorbed in the tubes was then removed, and they were then inoculated with pure cultures of *B. coli* and incubated for 48 hours at 40° C. in an atmosphere of CO_2 at atmospheric pressure. Control tubes were inoculated and incubated each time under ordinary atmospheric conditions. The results of these experiments are given below. Two classifications

are given for the tubes charged with CO_2; "A" being the last we have carried through, charged under somewhat more perfect conditions than "B," which represent the earlier work. "C" represents the control tubes, corresponding to the ordinary conditions under which fermentation tubes are incubated.

	A	B	C
Maximum percentage CO_2.....	78	63	40
Minimum " "	70	40	12
Average " "	75	49	30

It was proven by careful experiments that the high percentage of CO_2 obtained under these conditions was not due to the liberation of the CO_2 used in charging the tubes under the physical conditions of incubation.

We do not feel satisfied that we have produced conditions perfect enough to give us the exact CO_2 content; but the evidence seems to indicate clearly that for the class of cultures we have made use of in these experiments the percentage of CO_2 actually produced is approximately constant, and is probably at least 75 per cent of the total quantity of gas produced. And from the marked similarity of the curves showing the relations between gas and CO_2, for this class of cultures and for all the other classes given, it may reasonably be inferred that the same statement is of more or less general application.

(*c*) *Diagnostic value of the fermentation test.*—In a careful consideration of the diagnostic value of the fermentation test, attention must be given separately to its application to the presumptive and to the confirmatory tests, and also to its relative values when used qualitatively or quantitatively.

The presumptive gas test depends for its value entirely upon the principle that a sample of water properly inoculated into fermentation tubes, giving quantities of gas and CO_2 lying within certain limits, prescribed as the result of much careful experience, *most probably* contains *B. coli.* The fermentation test, in its application to the "presumptive test" is of an unquestioned value, and an essential feature of this is that it shall be quantitative.

It is common experience and is borne out by our own data, that the quantity of gas formed in confirmatory fermentation tubes varies within wide limits. We have shown by the foregoing data

that the percentage of CO_2 actually produced by *B. coli* and also by other gas-forming organisms, is approximately constant, and probably 75 per cent or more of the total gas; and that the variations from that quantity can in general be explained by the physical phenomenon of absorption of the CO_2 during incubation. We know further that the majority of cultures that show what may be termed "abnormal" quantities of gas and CO_2 in the subculture fermentation tube may be brought to such a condition, by proper methods of rejuvenation, that they will produce gas and CO_2 well within the "normal" limits—that is, approaching more or less closely to "about 50 per cent total gas, of which about one-third is CO_2" as measured by the ordinary methods.

If, then, the quantity of gas produced by a miscellaneous lot of organisms can be controlled, and the percentage of CO_2 is approximately constant, the diagnostic value of a *quantitative* gas test in the determination of the specific organism, *B. coli*, can be but small. As a *qualitative* test in this connection, it is of course of the greatest value.

Generally speaking, however, all the evidence we have seems to indicate that there is no determinative significance attached to the quantities of gas and CO_2 in the confirmatory or subculture fermentation tube.

As we have indicated below, our conclusions on this point have led us to eliminate all observations on gas formation except the simple presence or absence of gas. This permits the elimination also, of the old, cumbersome, expensive, difficultly cleaned fermentation tube, and the use of a gas tube improvised by inverting a small test-tube inside a larger one. We have made successful use of tubes of the following sizes:

Outside tube, $6'' \times 1''$
Inside tube, $2\text{-}3/4'' \times 1/2''$

The inside tube, which is simply placed in an inverted position in the larger tube, with the latter filled with the necessary quantity of broth, will fill on being taken out of the sterilizer. High steam pressure is not necessary. The tubes will fill equally well in the Arnold and in the autoclave.

This makes the examination of large quantities of water for gas formers a simple operation, too. It is only necessary to invert a

small test-tube inside an ehrlenmeyer flask holding sufficient broth to fill the inverted tube on sterilization, and of the proper strength. The sample of water is then added, and incubation gives visibly and unmistakably the presence or absence of gas.

(2) LENGTH OF PERIOD OF PRELIMINARY FERMENTATION.

The exigencies of a laboratory in which a large volume of work is carried on necessitate not only the elimination of unnecessary work, but also economy in such factors as time of incubation of cultures, quantity of glassware in use at one time, incubator space, etc. Some studies have therefore been made to show the value of a 24-hour period as compared with a 48-hour period of incubation of preliminary fermentation tubes.

Out of 1091 preliminary fermentation tubes showing approximately typical gas and CO_2 at the end of 48 hours only 519 tubes, or less than 50 per cent of them, showed quantities of gas falling within the proper limits for positive presumptive test at the end of 24 hours. It is plainly evident from this that a 24-hour period of incubation for the fermentation tube in the presumptive test is not sufficient.

A 24-hour period is sufficient, however, to indicate a negative result in case there is no gas formed at the end of that period, as the following figures will show:

Number of samples showing no gas in 24 hours, but some gas in 48 hours = 173.

Of these proven to contain coli = 2.

If all these 173 samples had been recorded as negative at the end of 24 hours when they did not show any gas, the error would have been only 1 per cent.

We may also conclude from these results, that when all suspected cultures are regularly carried through the complete test for coli, we may after plating from the preliminary fermentation tube to the litmus lactose agar plate at or before 24 hours, discard all the preliminary fermentation tubes, recording as negative all those which contain no gas at 24 hours. By so doing we lose the additional diagnostic value of the readings on gas and CO_2 in preliminary tubes, but these at best are of less value than the results obtained in subculture dextrose broth; and even these latter we have shown to be of but small value.

(3) VALUATION OF SUBCULTURE TESTS.

From an analysis of accumulated data such as we have here, the relative value of the various subculture tests may be determined.

These are values which of course will vary with a great many conditions, as will be seen from the tabulation given below. But the variation is over a comparatively limited range, especially in the case of those subcultural tests subsequent to the litmus lactose agar plate, and therefore the figures given are believed to have a more or less general value.

The test for *B. coli* is throughout a process of elimination. Were we carrying on our routine work entirely by the presumptive tests then we would eliminate by the first gas test all those cultures which, by the law of probabilities, were not positive. In applying the complete test for *B. coli*, however, we may avoid some of the uncertain factors of this application. We eliminate only those cultures which we have the best reasons to believe will be negative and proceed to confirm either positive or negative, by further work, all cultures which up to this point may be looked upon as suspicious.

This valuation of the subculture work is therefore based on a total number of cultures somewhat greater than would be considered probably positive on the strength of the presumptive gas test alone. From the tables it is evident that the greater part of this excess of doubtful cultures is eliminated by the very next test in order, the litmus lactose agar plate.

In these tables we have considered 100 per cent to represent the total number of preliminary fermentation tubes in which there was any gas formation in 24 hours, or if none in 24 hours, then an appreciable amount in 48 hours. The same matter is given in two forms; first, showing separately the values of the various subculture tests on the basis defined above; and second, showing on the same basis the percentage of cultures remaining after elimination by each of the successive subculture tests.

One complication must be noted. A good many cultures are eliminated through the failure of more than one of the tests, and these have been included under one head, as miscellaneous negatives.

TABLE 5.

MONTH	TOTAL NUMBER OF SAMPLES	PERCENTAGE OF SAMPLES GIVING PRELIMINARY FERMENTATION	NUMBER OF SAMPLES GIVING PRELIMINARY FERMENTATION	PERCENTAGE OF CULTURES SHOWING PRELIMINARY FERMENTATION, ELIMINATED BY FOLLOWING TESTS:							
				Litmus Lactose Agar Plate	Agar Slant and Microscopical Examination	Miscellaneous Subcult.	Nitrites	Indol	Milk	Gel. Stab	Confirmed Positive
March	411	39.4	162	53.1	9.3	7.4	2.5	3.1	2.5	0.6	21.5
April	350	58.0	203	41.4	3.5	7.4	5.4	1.0	3.0	0.5	37.8
May	432	47.9	207	82.6	1.4	2.9	4.3	1.4	1.4	0.0	6.0
June	424	70.5	299	54.2	3.3	10.4	1.7	6.0	3.0	0.0	21.4
July	285	79.6	227	47.1	6.2	11.5	4.8	6.2	3.1	2.6	18.5
August	487	85.6	417	33.3	7.2	4.1	2.2	9.4	1.4	1.2	41.2
September	278	67.3	187	48.1	14.4	8.0	1.1	8.6	4.8	0.5	14.5
October	330	61.8	204	48.5	4.4	7.8	1.5	2.5	10.3	1.5	23.5
November	275	45.1	124	46.8	0.7	4.8	4.8	16.1	0.0	0.8	17.0
December	281	50.2	141	29.1	14.9	10.6	11.3	6.4	0.7	0.7	26.3
Totals	3553		2171								
Mean values		61.1		47.7	6.8	7.3	3.5	6.0	3.0	0.9	24.8

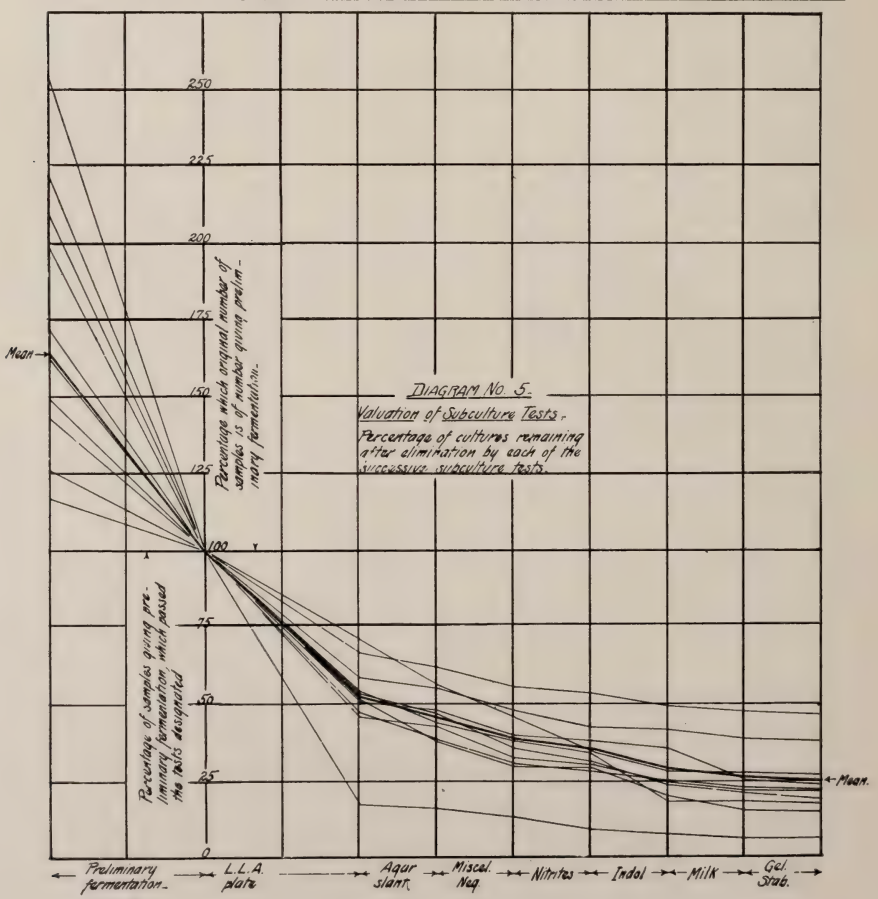
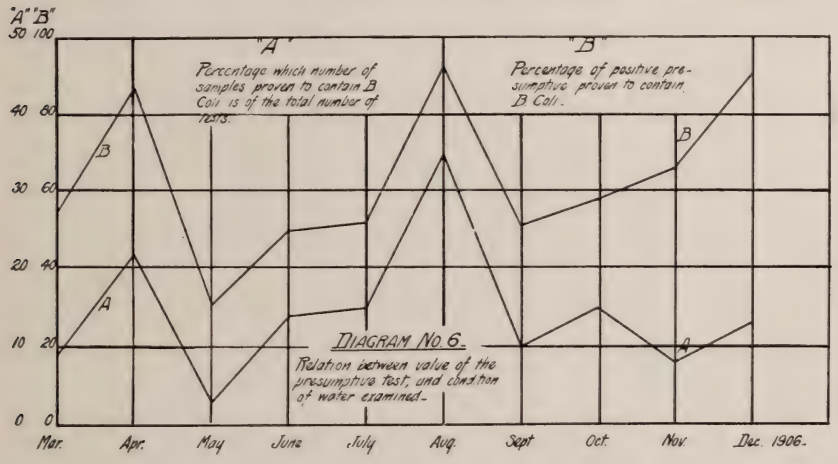


TABLE 6.

Month	Samples Giving Preliminary Fermentation	Percentage of Samples Giving Preliminary Fermentation, Passing All Tests to and Including						
		L. L. A. Plate	Agar Slant and Microscop. Examination	Miscellaneous Subcult.	Nitrites	Indol	Milk	Gel. Stab
March.....	100	46.9	37.6	30.2	27.7	24.6	22.1	21.5
April.....	100	58.6	55.1	47.7	42.3	41.3	38.3	37.8
May.....	100	17.4	16.0	13.1	8.8	7.4	6.0	6.0
June.....	100	45.8	42.5	32.1	30.4	24.4	21.4	21.4
July.....	100	52.9	46.7	35.2	30.4	24.2	21.1	18.5
August.....	100	66.7	59.5	55.4	53.2	43.8	42.4	41.2
September.....	100	51.9	37.5	29.5	28.4	19.8	15.0	14.5
October.....	100	51.5	47.1	39.3	37.8	35.3	25.0	23.5
November.....	100	53.2	43.5	38.7	33.9	17.8	17.8	17.0
December.....	100	70.9	56.0	45.4	34.1	27.7	27.0	26.3
Mean values	100	52.3	45.5	38.2	34.7	28.7	25.7	24.8



These tables and diagrams show that by far the greater part of the elimination of cultures is effected in the preliminary fermentation tube and the litmus lactose agar plate, subsequent eliminations being comparatively small and constant. Therefore the best presumptive test we could use for the conditions in this laboratory would be the preliminary fermentation tube followed by the litmus lactose agar plate.

(4) THE PRESUMPTIVE GAS TEST AND ITS APPLICATION TO THE ROUTINE EXAMINATION OF POTOMAC WATER, IN COMPARISON WITH A COMPLETE CONFIRMATORY TEST.

While the regular procedure of this laboratory in the determination of the presence of *B. coli* is to carry each culture through the full

confirmatory tests, it has been recognized as desirable to determine the value of the so-called "presumptive tests" on this water.

There is sometimes a misconception of the proper meaning of the term "presumptive test." A positive test of this kind is often construed to mean that *B. coli* is presumably present. There is not accuracy enough in this presumption. If would be better not to confuse the careful determination of the specific organism *B. coli* with the time and labor-saving determination, by means of this presumptive gas test, of the general character of a water as regards pollution. That the use of this test gives a reasonably accurate idea as to whether or not pollution exists, and in what quantity, has been conclusively proven, and is generally accepted. But the relation between the percentage occurrence of *B. coli*, and the percentage of samples giving presumptive evidence of pollution is very variable, and the one should not be taken for the other.

We have noted each culture during the preliminary cultivation with reference to the fulfillment of the following conditions:

(1) Total gas in 48 hours, between 25 and 70 per cent of the capacity of the closed arm of the fermentation tube.

(2) CO_2 between 20 and 40 per cent of the total gas.

Cultures having the above quantities of gas and CO_2 were considered typical positive presumptive.

Comparison of these figures with the total number of samples determined positive by the complete test is given below:

TABLE 7.

COMPARISON OF PRESUMPTIVE AND CONFIRMATORY TESTS ON POTOMAC WATER

1906	Total Separate Presumptive Tests	Total Positive Presumptive	Total Confirmed Positive	Percentage Which Confirmed Is of Total Samples	Percentage Confirmed Is of Presumptive
March	411	64	35	9	55
April	350	89	77	22	87
May	432	39	12	3	31
June	424	121	58	14	48
July	285	84	42	15	50
August	487	186	172	35	92
September	278	55	27	10	49
October	330	83	48	15	58
November	275	32	21	8	66
December	281	41	37	13	90
Totals	3,553	794	529		67

The figures we have given for the Potomac water show the great

variation in this relation under different conditions of pollution. The figures showing the occurrence of *B. coli* are the logical ones from which to judge of the sanitary condition of the water, and especially from which to compute efficiences of the various parts of the purification process, because they represent something much more definite than do the results of the presumptive tests. The latter, however, would be of value in the absence of the former, indicating with perhaps equal accuracy the variations in the polluted conditions of the water.

We have observed an interesting relation between the value of the presumptive test and the condition of the water under consideration. The condition of the water as regards pollution may be represented by the percentage which the number of samples proven to contain *B. coli* is of the total number of tests.

The value of the presumptive test for each month is given by the percentage of cultures determined as positive presumptive which are proven to contain *B. coli.*

These figures, when plotted, show a marked similarity in their variations, indicating that the value of the presumptive test as measured above varies with the quantity of pollution in the water, being least when the water is at its best, and greatest when there is the greatest quantity of pollution present in the water. This relation is indicated only for the particular class of water that comes under our routine examination, namely a surface water with only a moderate amount of pollution. Presumably the same relation would hold for any similar water not grossly and constantly polluted, but it would not hold for a water highly polluted with sewage.

(5) OTHER PRESUMPTIVE TESTS.

(*a*) *Direct plating on Wurtz litmus lactose agar plate.*—The Potomac water contains too little pollution for this method to give any satisfaction. The dextrose broth method is much more delicate, and we have discontinued attempts to get satisfactory indications by this means.

(*b*) *Neutral red reaction.*—Our trials of the neutral red reaction have given unreliable results. In these tests practically all the cultures confirmed positive have given the typical reduction of neutral red

in the fermentation tube. The converse does not hold true. Of all the cultures giving typical reduction of neutral red, in conjunction with typical gas and CO_2 only 67 per cent were confirmed positive. There was one isolated case in which a negative neutral red corresponded to a negative confirmatory test. This condition is the principal one which should lend value to the test, and in the absence of any considerable number of samples fulfilling this condition, we can only conclude that the value of the test for application to this water is questionable.

(*c*) *Use of the bile salts.*—The use of the bile salts and their acids has been recommended for the preliminary cultivation in the examination of water for *B. coli*. We have been and are still making a study of the value of media of this kind as applied to the Potomac water. There is unquestionably a marked restraining influence exerted by the bile salts and their acids, upon the growth of bacteria, and unquestionably, too, this restraining influence is selective. The statement has been made that they *favored* the growth of *B. coli*. This statement seems ill advised, and we think should be qualified in accordance with the following views.

The restraining influence acts upon all forms of bacteria; in greatest degree upon water bacteria and saprophytes of that class; probably in the least degree upon *B. coli* and allied forms whose natural habitat is the intestine where they are exposed to somewhat similar conditions.

Our own figures, and also the figures given by others in this connection, indicate that under conditions of considerable pollution there are decided advantages in the use of lactose bile. But the water we have to examine here in our routine work, including not only the filtered water but also the unfiltered water during the greater part of the time, contains so little sewage pollution that a strong inhibiting agent cannot safely be used without running the risk of checking the development of the occasional sewage organisms along with the saprophytic forms.

We can say without hesitation that the data so far obtained during a three-months' period of trial of Jackson's "Bile Lactose," in comparison with dextrose broth, do not warrant us in giving up the latter in favor of the former.

The value of the test with the bile lactose on unpolluted or slightly polluted water, such as we have to deal with the greater part of the time, is uniformly less than with dextrose broth, except in the larger quantities of water.

The ideal media for preliminary cultivation is one that always permits and never inhibits the growth of *B. coli* while it does inhibit the growth of some other organisms, preventing in as large a measure as possible the effects of overgrowth and antagonism. The bile lactose has too great an inhibiting effect for our conditions and the dextrose broth too little. Therefore we may look with some hope of success to find a medium more nearly ideal for our conditions, by combining the good points of the two in proper proportions, and it is in this direction that we are now carrying our studies on this subject.

REVISION OF THE METHODS OF THIS LABORATORY FOR THE DETERMINATION OF *B. coli.*

A review of the foregoing data has led us to adopt the following scheme for the determination of *B. coli* in Potomac water:

(1) *Preliminary incubation in ordinary dextrose broth fermentation tubes at 40° C. for 24 hours.*—If no gas forms in 24 hours, the test may be considered negative. If gas forms within 24 hours, those tubes showing gas shall be fished, and litmus lactose agar plate cultures made. The quantity of gas, presence of CO_2, and reaction after incubation have no determinative significance, and therefore need not be recorded.

(2) *Litmus lactose agar plate.*—The litmus lactose agar plate shall be incubated at 40° C. for 18 to 24 hours, 18 being usually sufficient. Those plates showing no development of red colonies in 24 hours are considered negative. From those upon which red colonies have developed, representative red colonies shall be fished, and incubated upon agar slants.

(3) *Agar slants.*—Agar slants shall be incubated at 40° C. for 24 hours, and if retained for a longer period shall be kept in the 20° incubator. Agar slant cultures which have typical cultural characteristics need not be examined microscopically, Atypical cultures shall be examined in the hanging drop before deciding to discard or carry through subculture. This examination should be made within 24

hours, and not later than 48 hours. Within 48 hours the pure cultures which have passed the agar slant shall be inoculated into subculture media as follows:

(4*a*) *Dextrose broth fermentation tube.*—These shall be incubated at 40° C. for 24 hours when presence or absence of gas shall be noted. Absence of gas shall be recorded as negative. The quantity of gas, presence of CO_2, and reaction need not be recorded.

(4*b*) *Milk.*—This shall be incubated at 40° C. for two days, and examined daily for coagulation, and digestion of the casein. Coagulation by itself is positive. Failure to coagulate or digestion of casein after coagulation shall be recorded as negative.

(4*c*) *Nitrate broth.*—This shall be incubated, together with a blank, at 40° C. for two days and shall then be tested for nitrites If nitrites have been produced, the test is recorded as positive.

(4*d*) *Peptone broth.*—This shall be incubated together with a blank, at 40° C. for three days, and shall then be tested for indol. If indol has been produced, the test is recorded as positive.

On account of the insignificant value of the test for liquefaction of gelatin in the examination of the Potomac water, and the large quantity of glassware kept continually out of service for this test, it may hereafter be omitted from our routine examinations, without introducing any appreciable error.

ON THE PHYSIOLOGICAL NATURE OF THE "GLANDULAR HYPERPLASIAS" OF DOG'S THYROIDS WITH A DETAILED REPORT OF A CASE TYPICAL OF THE GROUP.*

DAVID MARINE.

(From the Department of Pathology, Western Reserve University, Cleveland, Ohio.)

IN a previous communication[1] it was stated that 90 per cent of all the street dogs in the district of Cleveland showed on histological examination of the thyroids the so called "glandular hyperplasia." It was also stated that the most reasonable deduction from this observation was to consider this hyperplasia as a "Physiologic reaction to a deficiency." It was further stated that on purely anatomic grounds I had reason to believe that glands which once showed this hyperplasia had reverted to a more normal type, suggesting that in these cases the deficiency had been met. No suggestion was then offered as to what this deficiency might be, and it is in this connection that I wish to report a case and bring forward some deductions gathered from a review of the literature on thyroid affections.

This case typifies, I think, the general group of "glandular hyperplasias" in the dog, which I have reason to believe are cretinoid in nature. The dog came under observation October 18, 1906. No previous history was obtainable. The notes of the case as made from time to time are as follows:

The patient is a male, mongrel, bull-terrier puppy, probably five to six weeks old, and weighing roughly 2.728 kilos (5½ lbs.). He is somewhat emaciated and markedly pot-bellied, The bony prominences are distinct. The hair is tawny, dry, coarse, and without gloss. There is a constant overflow of tear-fluid which keeps both cheeks streaked with moisture. The eyes are prominent, but for the species not abnormally so. The thyroid lobes are markedly and apparently symmetrically enlarged. The veins over the goitre are distended, at times more markedly than at others, and definitely pulsate synchronously with the auricular systole. The thyroid lobes, each about the size and of the shape of a hen's egg, are moderately soft and have an expansile pulsation accompanied by a brûit. The tumors vary in size, evidently depending on the amount of blood they contain. They seem larger in the evening than in the morning. The puppy becomes easily fatigued, becomes quite dyspneic, and during these spells the thyroids are notably larger. There is a con-

* Received for publication March 9, 1907.

[1] *Cleveland Med. Jour.*, 1907, 6, p. 45.

stant fine tremor not relieved by heat. The legs are always cold. The mental condition is marked by apathy, sluggishness, and seeming indifference to his surroundings. The appetite is good; stools soft and unformed.

The dog was kept on a milk diet until October 28, but without any evident improvement. I wished to remove a lobe for a control and histological examination, but thinking the risk of operation too great at the time the following was administered:

October 28. 3 gtt. sat. alc. sol. iodine in milk.
October 30. 2 " " " " " " " .
November 1. 2 " " " " " " " .
November 2. 2 five gr. tablets, desiccated thyroid (Armour).
November 4. 2 " " " " " (").
November 6. 2 " " " " " (").
November 6. 2 gtt. sat. alc. sol. iodine in food.

November 7. There is a distinctly noticeable change in the dog's physical and mental condition. He is more active, cheerful, playful, comes at beckoning, and takes notice of his surroundings. The abdomen is less distended. The feces are soft but formed. The thyroid gland is quite firm, though the vessels are large and pulsation is felt. The heart rate has never been accelerated, but systole of the ventricles produces a distinct shock. Tremor still persists.

His condition was considered sufficiently improved to stand an operation and under ether the right lobe was removed. The gross appearance of the gland is as follows: The vessels of the lobe are very large and tortuous; the gland is extremely vascular and the capsule is slightly thickened. The lobes are joined across the midline by a band of thyroid tissue 1 c.m. wide $\times \frac{1}{2}$ cm. thick. (This condition is quite common in goitrous puppies; Halsted noted this in the litters of goitrous puppies which came to his notice.) On section the lobe is moist, soft, cellular, and grayish pink in color. A slight amount of thin colloid exudes. The acini are not visible. The gland measures $7 \times 4 \times 3.5$ cm. and weighs 28 grams. Microscopic appearance is given later. The dog stood the anesthetic well and rallied quickly, little ether being necessary. The hemoglobin seemed low. Milk diet.

November 9. Dog is active, cheerful, with appetite good. Heart rate has been rapid since operation.

November 12. 1 five gr. tablet desiccated thyroid, and 2 gtt. sat. alc. sol. iodine. Milk diet to which some bread is added and for the first time a little meat.

November 14. Puppy is mentally alert and playful. The skin is softer, but the hair, however, still dry and coarse. Wound has healed per primam, bandage removed.

November 15. 2 five gr. thyroid tablets.
November 16. 2 " " " "

November 19. The dog is given a bath and large quantities of loose, dead hair are removed, together with considerable desquamated epithelium. After removal of the dead hair a crop of fine, short, glossy hair is visible. One five-grain thyroid tablet given, and 2 gtt. sat. alc. sol. iodine. The remaining lobe is smaller and firmer than immediately after operation. No expansile pulsation. Dog is growing rapidly. Mentally he appears to be a normal dog. Body temperature is higher, extremities are warm. Heart rate is still rapid. Fine tremor has disappeared.

November 22. One five-grain thyroid tablet; the dog, continues to shed dead hair and epidermis and the fine glossy coat is now quite in evidence.

November 28. Hair and skin normal; growing fast; appetite good.

December 11. Growing rapidly; mentally and physically a normal dog save that the heart is still somewhat rapid and the impulse forcible. The gland is firm, pulsation absent, distinctly reduced in size.

This afternoon a "V"-shaped piece of the remaining lobe was removed for microscopic examination. The tissue is quite vascular, pink, translucent gray in color, and firm to the touch. The acini are visible and their colloid content appears normal.

December 17. Dog seems perfectly normal. Wound has healed. Diet of bread milk and a little meat.

January 14, 1907. Killed. Weight 6.2 kilos (12.5 lbs.), in excellent condition, the usual repositories containing normal amount of fat. A single accessory thyroid is found on the aorta just above the pericardial reflection. The heart is enlarged. The blood is of normal color.

The remaining lobe of the thyroid contains a large amount of clear syrupy colloid. Its vascularity is increased. The gland is firm, hard, yellowish gray, and translucent. The vesicles are distinct, variable in size, and all distended with colloid. The lobe weighs 27.1 grams, showing that after removal of the blood, the lobes practically had the same weight.

At the time of death, the dog, clinically, appeared normal in every way save that the thyroid lobe was enlarged and the heart-beat was forcible.

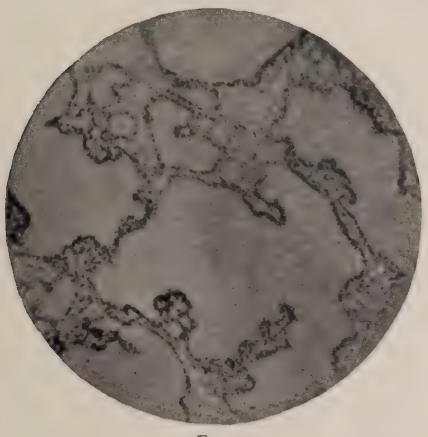


Fig. 1.

To summarize: We have a small puppy weighing roughly 2.728 kilos (5½ lbs.) on October 28, 1906. Roughly, 90 mg, of a saturated alcoholic solution of iron and 14 five gr. thyroid tablets were given from October 28 to November 22, after which date he received no thyroid and no iodine other than what might have been obtained from the food. This puppy on admission was anemic, apathetic, pot-bellied. His hair was dry and coarse, eyes weeping; he had a large symmetrical swelling of the thyroid and expansile pulsation, accompanied by a brûit. These symptoms practically cleared up in 25 days, and in the space of 2½ months the dog gained 3.7 kilos (7½ lbs.), and became physically and mentally a normal dog save that the thryoid was still enlarged and the heart impulse forcible. The microscopic appearance of the three thyroid specimens is as follows:

(1) Specimen 1 (D–185a), removed November 7, 1906 (Fig. 1).

There is a slight general increase in the fibrous framework with band-like accentuations supporting the larger vessels. The colloid is palely staining with here and there slight vacuolation. The acini are irregularly enlarged and distorted by intra-acinar projections and invaginations of the lining epithelium. The epithelium is in general columnar; occasionly high cubical areas are noted, and over the most of the crests of the intra-acinar projections it is raised to the high columnar type. The nuclei are large, pale, vesicular, and basal. There are in places crops of apparently newly formed acini. (This variable condition of the epithelium is best explained by supposing that the return to the normal type had started already, and that had the tissue been examined before medication or the appearance of signs of clinical improvement the epithelial cells would have had the usual (glandular) uniform columnar appearance.)

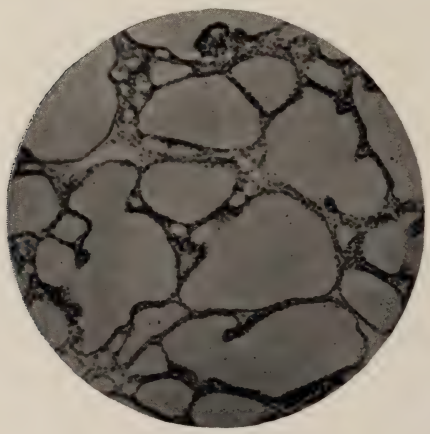


FIG. 2.

We have then a highly vascular gland with increase in the fibrous tissue, reduction in the stainable colloid, moderate papillomatous ingrowths, and invaginations of the epithelium which varies from high cubical to definitely columnar cells, and all showing large, pale, basal, round, vesicular nuclei.

(2) Specimen 2 (D—185b), removed December 11, 1906 (Fig. 2).

The stroma is quite compact, producing a notable decrease in the prominence of the fibrous tissue framework. The colloid is dense and uniform. The epithelium is generally low, cubical, except over the intra-acinar projections where it still retains a columnar type. The nuclei are generally small and deeply staining. There is no prominence of the vessels and no desquamation. The appearance now is that of a colloid adenoma, except for the occasional tufts of columnar epithelial cells capping the intra-acinar projections.

To sum up, we have four striking differences from "Specimen 1": (1) reduced prominence of the fibrous framework; (2) a nearly complete disappearance of the columnar epithelium, with return to a low cubical type; (3) a change from the large, pale, vesicular nuclei to small, deeply staining ones, and (4) an increase in the stainable colloid.

(3) Specimen 3 (D—185c), removed January 14, 1907 (Fig. 3).

The acini are large and filled with dense, uniformly staining colloid which rounds out the walls and compresses them to thin partitions, while the intra-acinar projections are compressed to cordlike masses covered with single layers of low cubical epithelium. The epithelial cells are everywhere low (flat), cubical, with small, deeply staining nuclei. Colloidlike material is visible in the lymphatics. The fibrous framework is compact and seemingly greatly decreased.

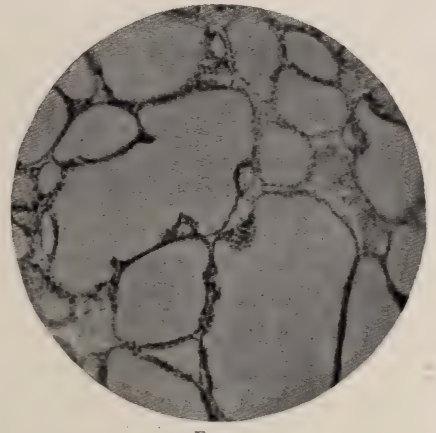


Fig. 3.

The picture now is like that seen in the pure colloid adenoma, save that the intra-acinar twigs are more numerous, and it is exactly like the picture observed in the instances where there was reason, purely from anatomical grounds, to suspect that there had previously been glandular hyperplasia.

There is now practically normal types of gland cell, nucleus and colloid, with large globular acini, into which project sprigs reduced almost to lines by their epithelial covering returning to the flat cubical type. The picture might be described as a simple adenoma or colloid goitre.

This case typifies what I had reason to suspect, purely on anatomical grounds, namely that this glandular hyperplasia may and does revert under certain conditions to a normal type of gland. In the routine examination of 250 dogs' thyroids I had seen conditions that at the time were considered "varying degrees of glandular hyperplasia" in conformity with the term suggested by Halsted and others, the vast majority of which seemed in the progressive stage, but a few, while still hyperplastic, seemed to be regressive, i. e., the hyperplasia was not universal. Indeed in a single acinus the epithelium of the main wall would be nearly normal, while that of a papillary invagination would show a columnar modification, and in other adjoining acini, all the cells would be columnar. Then, there were a few glands, enlarged, containing dense colloid, but whose acini showed many sprigs of tissue covered with flattened epithelium that seemed to be the remnants of former intra-acinar invaginations.

It was from these observations that I suspected a deficiency in those cases (90 per cent) which showed the progressive glandular change, and that the deficiency had been met in those few (4.5 per cent) cases which showed the regressive change. With this clew I began to inquire what this deficiency might be due to. Naturally iodine first suggested itself. But despite the enormous literature bearing on the relation of iodine to thyroid affections, both before and since Bauman's discovery, I can find no references in the available literature to instances in which these anatomic changes had been followed carefully with the microscope parallel with the administration of iodine or thyroid products.

The relation of the administration of iodine to these anatomic changes is as yet in part a weighing of evidence and a casting of opinion. In the first place one is confronted with the following questions: (1) Did the dog recover in spite of iodine medication? (2) Was his recovery hastened by the administration of iodine? Relative to the first question, it is the generally accepted opinion that iodine compounds benefit the majority of simple goitres, whether in man or in animals. (We are dealing here with simple goitre only.) Kocher states that 90 per cent of all goitres may be so improved by the administration of iodine-containing compounds as to render operation unnecessary. To show that spontaneous recovery frequently occurs we have only to recall those forms associated with puberty and pregnancy. In most of these cases it subsides. Then too we should recall in this connection the cases that have been observed in dogs where there was a return to a normal type of gland without clinical disappearance of the goitre.

It might be well here to add that when these "glandular hyperplasias" revert to a more normal type following the administration of iodine compounds, their acini, which are always enlarged, do not reduce in size, but appear even larger. This is readily seen to be so, for when a vascular, cellular acinus is stripped of its columnar epithelium and increased blood supply, its intra-acinar space becomes larger. Now while the experimental evidence shows that there is a slight gradual reduction in the size of the acini, and hence in the size of the gland as a whole, it is not probable that they could revert to a normal size; i. e., reduce in volume 20–30 times. In

other words there is every reason to believe that while iodine compounds reduce the hyperplasia—relieve the deficiency—there is but moderate reduction in the size of the gland. This reduction, in dogs, varies from $\frac{1}{4}$–$\frac{1}{2}$ the original volume, depending on the duration of the hyperplasia and in consequence the amount of fibrosis, and also on the age of the animal, the young, as is well known, reacting much more rapidly.

Thus the removal of the etiological factor in the production of the goitre would in nowise entail a relief from the swelling if it be well advanced, even though there are no complications. Indeed, in the dog at least, it would rather compel us to suppose a hyposecretion with a gradual absorption of the stored colloid.

The second question, as to whether iodine aided the anatomic changes, has in its favor a vast accumulation of circumstantial clinical evidence. (A critical review of this evidence will be published later.) Experiments with controls are now in progress that would seem to confirm experimentally this clinical experience and establish an anatomic basis for it, at least in the dog. The relation of the degree of "glandular hyperplasia" to the iodine content has of course interested me for some time, as it was early suspected that the iodine content might be reduced in those cases showing the hyperplasia. Through the courtesy of Dr. W. W. Williams I am able to report that the thyroid per gram weight removed at autopsy from the animal here described showed an enormous increase in the iodine content over the normal. I believe that iodine was stored in the gland, as has already been shown by many investigators to be the case in the human thyroids, following the administration of iodine. Unfortunately the iodine content of the first lobe could not be determined. From what we now know of thyreo-globulin and of Oswald's work on the iodine content of human goitres, there is reason to believe that the body economizes iodine not unlike it does iron, and indeed, carrying the analogy farther, there is reason to suspect that iodine is related to endemic goitre not unlike iron is to chlorosis. Both conditions are probably highly complex. But these two elements may be the nuclei around which the metabolic complexities of goitre and chlorosis are grouped.

In reviewing the literature one is struck with the multiplicity of

factors adduced from time to time as causal agents in the production of endemic goitre, most of which are probably without foundation. In general the views may be divided into (1) infectious; (2) metabolic. From a careful study of the writings of those most prominently engaged in the study of goitre a few deductions may be included here:

(1) That new goitrous districts are not arising apart from newly settled districts, and then only in the altitudes: in other words goitre districts remain singularly distinct.

(2) That goitrous districts are becoming less goitrous, and many previously goitrous districts are now free from goitre.

(3) That in general endemic goitre is proportionately much more prevalent among the poorer classes.

(4) That the so-called epidemics of goitre do not occur outside of goitre districts, and then among newly arrived subjects, whether man or animals.

(5) If water be associated with the cause of endemic goitre it seems rather to be due to the absence than to the presence of any particular substance in the water.

(6) Boiling the drinking water is said not to prevent the occurrence of goitre in goitrous districts.

(7) There is no record of goitre being endemic along the sea-coast.

(8) Cretinism associated with atrophy of or absence of the thyroid is about as frequent in non-goitrous as in goitrous districts, while in goitrous regions probably over 60 per cent of the cretins have enlarged thyroids.

(9) While all cretins seem to improve upon the administration of iodine, those with atrophy or absence of the thyroid do better with thyroid feeding, while those with goitre improve about as much with iodine alone, suggesting that in the one case we must supply the formed product, while in the other case the gland is able to metabolize its own product from the elementary substance.

(10) According to the case reports myxdema rarely precedes, occasionally accompanies, and frequently follows the symptom-complex of Graves' disease.

(11) It seems that the normal thyroid, like other normal organs, is more than sufficient to meet the normal physiologic needs.

(12) I have been able to find but two cases of myxdema (non-

operative) associated with tetany, suggesting that the parathyroids are relatively independent structures, thus bearing out the experimental evidence.

(13) There is no evidence for considering goitre other than a manifestation of or a reaction to a more general disorder.

(14) Carefully prepared histological data as to the occurence and incidence of "glandular hyperplasia" in animals, both in goitrous and non-goitrous districts, are not available, but would be of the greatest value.

In concluding this report the following points may be emphasized: The histologic changes described are too striking and too rapid to be considered accidental; That lack of iodine was the essential deficiency and iodine when supplied quickly overcame the needs; that there is reason for believing that the body economizes iodine not unlike it does iron, and that it is essential to normal metabolic activities; that the determination of the relation of the "glandular hyperplasias" to the iodine content is extremely important.

This relation can be predicted from the following data: Oswald has shown that, for man and calves, the iodine content in general varies directly with the amount of colloid, and I have observed that the glandular hyperplasia in dogs varies inversely to the amount of stainable colloid; therefore the iodine content should vary inversely to the degree of glandular hyperplasia. It seems that these glandular hyperplasias are common in dogs, cattle and sheep throughout the Great Lakes District.

I wish to thank Doctors C. H. Lenhard and M. B. Bonta for their services in the operative work, and also Doctors G. W. Crile and Torald Sollmann for many favors.

OBSERVATIONS ON THE STAINING OF ENCAPSULATED BACTERIA WITH PARTICULAR REFERENCE TO PNEUMOCOCCI AND STREPTOCOCCI.*

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IN the past few years quite a number of contributions to methods of staining encapsulated microorganisms have appeared in the literature. Yet there exists no lack of disagreement amongst the workers in this field both as regards the advantages of the various methods that have been proposed and as to the morphology of pneumococci and streptococci. It is the purpose of this paper to help clear up some of the fallacious ideas that have been put forth, and to give the results of experiences gathered in the laboratory of Mt. Sinai Hospital during a period of about three years.

The procedure for capsule staining that has given us the most satisfactory results appeared in a previous publication[1] where it was thus described.

The necessary solutions are as follows: (1) Müller's fluid (bichromate of potassium 2, 5 grms.; sulphate of sodium, 1.0 grm.; water, 100 c.c.) saturated with bichloride of mercury (ordinarily about 5 per cent). (2) Beef, human, or other blood serum diluted with an equal amount of normal salt solution; or ascitic or pleural fluid. (3) 80–95 per cent alcohol. (4) Tincture of iodine, U. S. P. (5) Freshly prepared stain;[2] anilin water gentian violet, made up as follows: anilin oil 10; water 100; shake, filter, and add 5 c.c. saturated alcoholic solution of gentian violet. (6) Two per cent watery salt solution.

The culture is thinly and carefully spread on a perfectly clean cover-slip by means of a drop of diluted serum. Just as the edges begin to dry[3] the fixing fluid, solution No. 1, is poured on, the cover gently warmed over the flame for about three seconds, rapidly washed in water, flushed once with alcohol, and then treated with iodine for one to two minutes. The iodine is in turn thoroughly washed off with alcohol, and the specimen dried in the air. Staining for two seconds, and washing with salt solution completes the procedure. The specimen is mounted in the salt solution and ringed with vaselin.

In order to attain uniform results—and this in our experience holds true with other methods of staining—a serous fluid of the

* Received for publication March 28, 1907.

[1] *Med. News*, 1904, 85, p. 1117; also *Centralbl. f. Bakt.*, 1905, Orig. 39, pp. 216, 335.

[2] A quantity sufficient to last for about four days ought to be prepared.

[3] Good results may even be obtained after the cover-slip preparation has been allowed to dry in the air; however, the method described is preferable because it gives much clearer fields.

proper quality must be selected. It has been our custom to employ sterile ascitic fluid which has been tested on typical pneumococci and to preserve some of it for capsule staining. A small quantity (25 c.c.) will suffice for a year's work.

Since the serous fluid has for its object the preservation of the capsules, the culture must be well impregnated with the drop on the slide before spreading. This is especially important in the preparation of stains of streptococci. A tiny bit of the culture, therefore, must be gently mixed with the drop of serum and then spread, taking care not to drag the organisms beyond the limits of the fluid. This mixing process is important when we have to deal with certain mucoid bacilli and *Strept. mucosus capsulatus*. Here, when the growths are very viscid, it is advantageous to make an emulsion in a few drops of serum on a separate slide, and then to transfer some of it to the cover-slip.

The fixing fluid should not remain on the cover-slip longer than three to five seconds; at about this time the specimen becomes opaque, an appearance that indicates the completion of the fixing process.

It is essential, furthermore, to make a practice of examining for capsules only on the most favorable media. These are the glucose-serum agar and a well-prepared Loeffler's coagulated serum. In our routine work we rely upon a 0.5–2 per cent glucose-serum agar made up with ascitic fluid, the latter constituting about one-fourth of the volume of the medium.

If we employ this method on a large number of pneumococci, streptococci, and encapsulated bacilli, we meet with certain regularly defined types of capsules. One of these is characteristic for the pneumococcus, another for certain streptococci, and still another for mucoid streptococci and mucoid bacilli—and so we may distinguish the following types:

1. The pneumococcus type.
2. The streptococcus type.
3. The mucoid type.

The pneumococcus type.—This type which is found on the most favorable culture media is illustrated by Fig. 1.[1] Here the diplococcus

[1] I am indebted to Dr. F. S. Mandlebaum, Director of the Laboratory, for the microphotographs reproduced in these pages.

is surrounded by a deeply staining, sharply defined, elliptical band, situated at some distance from the body of the bacterium and separated from it by a clear area. This clear interspace is sometimes stained but usually less deeply than the limiting membrane. The capsules have an elliptical outline and when they surround chains show distinct constrictions between the diplococci.

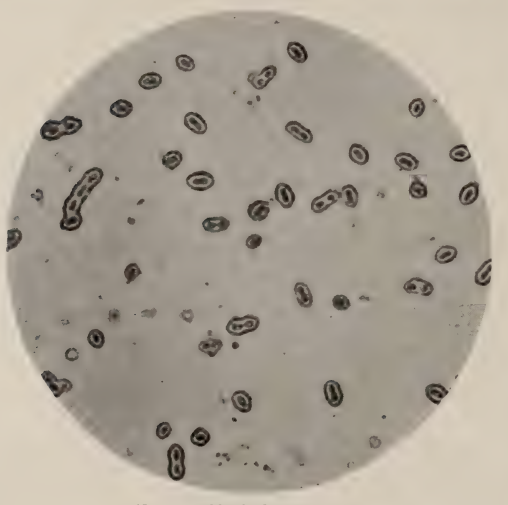

FIG. 1.—Typical pneumococcus.

When the conditions for growth are unfavorable, or after a number of transplantations, or at times in the very first cultures from the blood of the patients who have harbored pneumococcus for a considerable period of time, these typical appearances may be absent. The capsule degenerates, becomes narrower, and assumes the type characteristic for certain streptococci, or may even be entirely lost (see Fig. 2.). The strains vary considerably as to the length of time that the typical or well-matured form is retained. By subinoculations on very favorable media the usual form may often be restored. This also may be effected by inoculation into susceptible animals (mouse and rabbit).

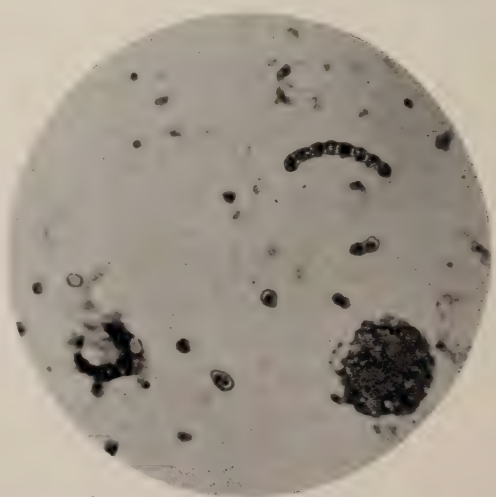

FIG. 2.—Degenerate pneumococci in an old empyema exudate.

In a study of a large number of strains of pneumococci, it was found that whenever the typical forms were present, the organism presented the biological and cultural features peculiar to the pneumococcus. Such organisms fermented inulin, although with some strains this did not occur regularly in every generation. Thus we consider the morphological characteristics of considerable importance in diagnosis.

The streptococcus type.—If we stain a large number of strains of pathogenic streptococci we note that there are some forms which show no capsule under any conditions, others which possess narrow capsules, and finally those that belong to the group *Strept. mucosus capsulatus*. The streptococcus type is shown in Fig. 3, an organism isolated by blood-culture from a patient suffering from a rather chronic form of streptococcemia. The capsule is narrow, the periphery rather close to the body of the organism (usually even more closely adapted than in the figure), and frequently shows constrictions separating either the single elements or the pairs of hemispherical elements when such are present. Pneumococci, when they degenerate both in culture or in old exudates, frequently have such capsules. They cannot then be differentiated morphologically from streptococci.

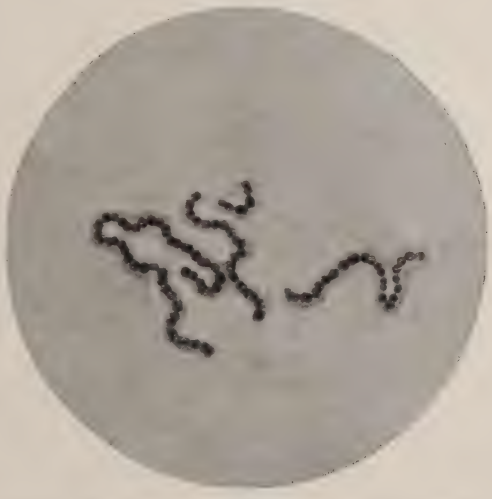

FIG. 3.—Streptococcus with the streptococcus type of capsule.

Fully 25 per cent of the pyogenic streptococci received in the laboratory in the course of routine bacteriological work show capsules. With a little experience it is not a difficult matter to predict whether a streptococcus will show a capsule or not, by observing the character of the growth on glucose-serum agar. Many streptococci, such as

those usually present in the feces, in the milk, and also some pathogenic varieties, show dry, often whitish, colonies. These are not encapsulated. Others, however, show moist watery colonies, and still others have even a mucoid appearance, particularly in the first generations. Such organisms regularly have the streptococcus type of capsule.

In a paper on *Strept. mucosus capsulatus*[1] I drew attention to the importance of differentiating some of these streptococci from *Strept. mucosus*, as follows:[2]

> There are streptococci which have a rich watery growth on serum agar and glucose serum agar, but that do not belong to the group *Strept. mucosus capsulatus*. These were found in the blood of cases of "ulcerative endocarditis," as well as in various local infectious processes. The colonies resemble those of the *Strept. mucosus* very much. When stained with the author's method they show the narrow "streptococcus type" of capsule. On glucose-serum agar they cause precipitation whereas the *Strept. mucosus* does not. Twenty-four hours after isolation from the blood of a patient, the colonies on the plates may be like those of *Strept. mucosus*. Later however, they cause distinct hemolysis, whereas the green color and absence of hemolysis are characteristic for *Strept. mucosus*. Such streptococci do not form a mucoid exudate when inoculated into animals, nor do they show the typical morphology of *Strept. mucosus* in the tissues or in the blood. They usually lose their watery appearance after a few transplantations.

None of the strains that I have examined fermented inulin.

The mucoid type.—Strept. mucosus capsulatus possesses a large capsule that stains diffusely and whose periphery has a tendency to be irregular and less well defined than that of the pneumococcus. It surrounds the diplococci or chains usually without showing any constrictions between the elements (see Figs. 4 and 5). Similar capsules are to be seen on certain strains of the Friedlander bacillus and of *M. tetragenus*.

Wadsworth[3] in a recent publication gives a critical review of some of the methods of staining encapsulated organisms and suggests a new method in which formalin is employed as a fixative. A similar method was tried by me several years ago.[4] I stated then that formalin was less reliable than the Müller-bichloride fixation. To quote, "Auf andere Fixationsmittel, wie Formalin und Flemingsche Flüssigkeit,

[1] Buerger, *Centralbl. f. Bakt.*, 1906, 41, p. 511.

[2] Translation.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 610.

[4] *Centralbl. f. Bakt.*, 1905, Orig. 39, p. 219.

habe ich verzichtet, da dieselben kein klares Gesichtsfeld gaben und überdies vor der oben empfohlenen Lösung keinerlei Vorteile voraus hatten." For the staining of organisms with very well-developed capsules the method of Hiss and the formalin fixation give excellent results and are simple and reliable. However, where the capsules are poorly developed, where the degenerative forms make their appearance, and in the case of streptococci not of the *mucosus* group, the method of Hiss fails to show capsules, and the formalin method gives varying results.

FIG. 4.—*Streptococcus mucosus capsulatus.*

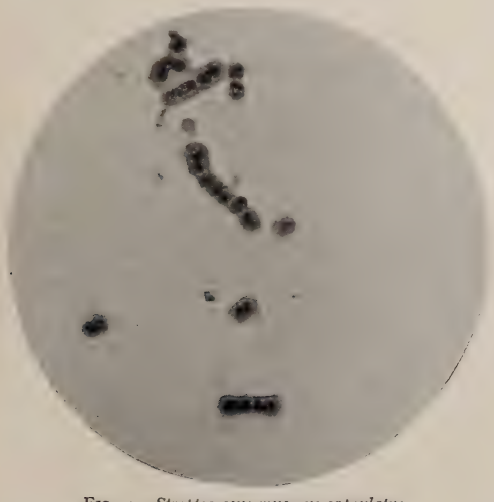

FIG. 5.—*Streptococcus mucosus capsulatus.*

In speaking of my method Wadsworth says, "Buerger fixes the smear of encapsulated pneumococci in Müller's fluid saturated with bichloride for one half-minute, and washes in water." In the articles in which the method is described and which he quotes, it is distinctly stated that the fixative is to remain on the coverslip for *three seconds* over the flame. The uniformly good results obtained in our laboratory by following out these directions make the footnote which Wadsworth adds—"In my experience

with this method longer exposures are advisable; in fact, essential" —very difficult to understand.

As regards the combined Gram and capsule stains of organisms in culture, Wadsworth is in error when he says, "With these methods of Smith and Buerger the bacterial cells, under favorable conditions, may be stained by the Gram stain and demonstrated with capsules." Smith's stain was only used in the staining of sputum or exudates. With the combined method, as first proposed by me, we have never failed to demonstrate a Gram negative capsule and a Gram positive cell body on pneumococci that showed capsules by the simple stain.[1]

Earlier, in this paper, I dwelt upon the question of capsules on streptococci, because this matter seems to be still a mooted one. Thus Wadsworth says, "Streptococci, in my experience, have rarely given definite capsules with these formalin methods," and in a footnote, "Occasionally a faint, hazy periphery, suggesting a shrunken or degenerated, partially dissolved capsule, was noted." It is hard to reconcile these statements with my own results. I have been able to demonstrate capsules on quite a large number of strains of streptococci with the formalin method, but more frequently with the Müller—bichloride of mercury fixation. As I have said before fully 25 per cent of streptococci examined in the laboratory, showed the "streptococcus type" of capsule. The formalin method gave results in a large number of these, the Hiss method rarely.

When we consider Wadsworth's failure to get the best possible results with both my own and the formalin method we can see the *rationale* for his statement, "The morphological differences of the capsules of the pneumococci, as compared with other encapsulated organisms resembling the pneumoccocus, which Buerger obtained with his simple stain, and upon which he lays so much stress, depend chiefly upon the varying stages of development or degeneration and solution, and upon the degree of decolorization. They are in no sense differential." The morphological types which I have pointed out represent not, as he would have it, stages of degeneration, but the highest developmental forms of the various organism. Pneumo-

[1] The combined capsule and Gram stain is carried out as follows: Spread in serum; fix with Müller bichloride of mercury (3–5 seconds); wash with water; tincture of iodine (1–2 min.); alcohol, dry; anilin water gentian violet (5–10 per cent); drain; Gram's iodine; drain; alcohol; water; aqueous fuchsin 10 per cent (2 min.); water; mount in water.

cocci when grown under favorable conditions show the pneumococcus type. Streptococci with capsules present these only under similar conditions and may lose them in subcultures. But the streptococcus, the type with the capsule, represents the most mature form of that organism. Whoever has demonstrated it on luxuriantly growing strains, and has missed it in later generations, will recognize the correctness of this assertion. Apparently the development of the streptococcus capsule is not as high as that of the pneumococcus.

In previous communications I discussed at length the differentiation of *Strept. mucosus* from the pneumococcus. Wadsworth thinks that morphological distinctions cannot be made. It is difficult to understand this viewpoint particularly when we glance at the differences shown in Figs. 1, 4, and 5. Still in another place he says, "The methods of Guarnieri, of Welch, and of Buerger, reliable, though in one way or another complicated, all give temporary mounts, and are thus unsatisfactory." Both the Welch and my method can give permanent mounts, specimens of which I have had in my possession for more than two years. It is true that these are inferior to the fresh mounts. For our purposes of diagnosis, however, it is far more important to be able to differentiate pneumococci from streptococci aided by reliable staining methods than to add permanent specimens to our collection.

It may be justly contended that the appearances described by me as typical for various encapsulated organisms are to some extent fashioned by the material employed in staining. Thus the bichloride of mercury in the fixative may, it is true, be responsible for a certain amount of shrinkage of the capsule substance. However, artificial appearances of some kind or other are produced by all methods. The serum used for the spreads may also have some influence on the nature of the capsule. For routine work, where it is important to obtain appearances that are of value in differentiation, the method adopted by us seems to be the most satisfactory.

ON THE PRESENCE OF HEMOLYTIC SUBSTANCES IN EDIBLE FUNGI.*

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IT has recently been pointed out by Abel and Ford[1] in a paper dealing with a chemical analysis of the constituents of *Amanita phalloides* that two poisons are present in this fungus, and that they may be separated by precipitation with ethyl alcohol. The alcohol precipitate contains the hemolytic substance originally described by Kobert[2] under the name "phallin," while the alcohol filtrate contains the Amanita-toxin found in the "deadly Amanita" by Ford[3] which is possibly but not certainly identical with a poisonous substance described by Kobert[4] and which he believed to be an alkaloid. It was also shown by Abel and Ford[5] that this hemolytic substance is not a "toxalbumin," as stated by Kobert, but a glucoside extremely sensitive to the action of heat, acids, and the digestive ferments. From these properties the authors conclude that this hemolytic glucoside can play no rôle whatever in intoxications by *Amanita phalloides* in man, since in these cases the cooked fungi are introduced into the stomach as food. The active principle is therefore the heat-resistant Amanita-toxin contained in the alcohol filtrate. It had already been suggested by Kunkel[6] on the basis of his pupil Seibert's[7] work, that phallin could not be the poisonous constituent since it was absent from dried specimens of *Amanita citrina*, a toxic species either closely related to or identical with *Amanita phalloides*. Finally Kobert[8] has recently receded from his earlier position in the matter, stating

* Received for publication March 16, 1907

[1] *Jour. Biol. Chem.*, 1907, 2, p. 273.

[2] *St. Petersburger med. Wchnschr.*, 1891, 16, pp. 463, 471.

[3] *Jour. Exper. Med.*, 1906, 8, p. 437.

Sitzungsber. d. naturf. Gesellsch. zu Rostock, 1899; also *Lehrbuch der Intoxicationen*, Stuttgart, 1906, 2, p. 625.

[5] *Loc. cit.*

[6] *Handbuch der Toxicologie*, 1901, 2, p. 1048.

[7] *Inaug. Dissert.*, München, 1903.

[8] *Lehrbuch der Intoxicationen*, 1906, 2, p. 763.

that phallin is by no means always present in the fungus, the active principle of which he thinks is still a matter for investigation.

In view of these various opinions it became a point of importance to study fresh specimens of *Amanita phalloides*, to determine whether hemolysins are always present in them and whether they disappear on drying, and finally to ascertain if similar blood-laking substances may be found in other fungi. This work was undertaken during the summer of 1906 at the Marine Biological Laboratory at Woods Hole, Mass., and I should like here to express my indebtedness to the acting director, Dr. Frank Lillie, and to the botanist, Dr. Moore, for many courtesies extended to me during the progress of the investigation. The pine woods in the vicinity of Woods Hole and on the adjacent islands abound in fungi of all sorts and descriptions, and an excellent opportunity was thus presented for studying perfectly fresh specimens. A large amount of material was also collected and dried, a subsequent study of this material being made in the Bacteriological Laboratory of Johns Hopkins University. The most important facts brought out during this study relate to *Amanita phalloides*, and to two closely related species of Amanita, *Amanita rubescens* and *Amanita solitaria.*

AMANITA PHALLOIDES.

Typical specimens of *Amanita phalloides* are in many cases quite devoid of hemolytic activity. A large number of individual plants were tested upon rabbits' and hens' blood corpuscles, both of which are sensitive to the action of the hemolysins in this fungus, and in many instances no laking of the corpuscles could be demonstrated. In other plants hemolysins were found in their usual strength, the distribution of these substances being seemingly independent of the locality in which the fungi were collected. In agreement with Kobert,[1] therefore, we must look upon this hemolytic substance as by no means constantly present in the "deadly Amanita," and on this account alone it could not be considered the active principle. Still more important facts were brought to light by the study of two other fungi, both of which are considered edible, one of which is powerfully, the other moderately, hemolytic.

[1] *Loc. cit.*

AMANITA RUBESCENS.

This is a very large Amanita which was especially abundant during the wet season in July. In appearance it is a typical Amanita. It has a widely expanded pileus of a dull-red color, covered, especially in the early stages, with thick, yellowish scales. The under surface of the pileus, the spores, the gills, and the stalk, are pure white, as well as the veil and poison cup.

As soon as the plant is touched or handled, however, the bruised flesh assumes a dull-red color, and the coloration is the most important means of recognizing the species. The change in color occurs within a few minutes, and by the time the plant has been taken to the laboratory but little of the original white is seen. The juice from the macerated fungus is of a dull-red color, while the dried specimens have this color markedly accentuated. Only when the plants are very young do they retain their typical appearance. The *Amanita rubescens* is considered edible by the majority of mycologists, Atkinson, and others, the only objection to its use for the table being its resemblance to *Amanita phalloides*. As far as the literature shows it is never the cause of poisoning.

When tested upon rabbits' and hens' corpuscles the juice of *Amanita rubescens* is powerfully hemolytic. Solution of the corpuscles takes place rapidly within 15 minutes to an hour, and in a far greater dilution than in any specimen of *Amanita phalloides* I have ever seen. The action of the hemolysins is similar to that of the "deadly Amanita," solution taking place without agglutination. Specimens of *Amanita rubescens* dried for several months and then tested are found to have retained their hemolysin unaltered in strength. It is completely destroyed by exposure to a temperature of 70° C. for half an hour, and the extracts so treated contain no Amanita toxin. When passed through a Berkefeld filter and heated to 70° C. they may be given subcutaneously in large doses to both rabbits and guinea-pigs without producing anything more than a transient edema at the site of inoculation. Chemical investigation of *Amanita rubescens* confirms the results of biological experiment. If the aqueous extract of this fungus be evaporated *in vacuo* at 35° C. to a small bulk and then precipitated with ethyl alcohol, the precipitate thus obtained contains the hemolysin. If the alcohol filtrate be evaporated *in vacuo* at 35° C.

to dryness and then taken up in water, it contains no toxin. This fraction may be administered in large quantity to both rabbits and guinea-pigs with practically no effect except a slight subcutaneous edema. The *Amanita rubescens*, therefore, while containing powerful hemolysins, contains no Amanita toxin, i. e., none of the active principle of the "deadly Amanita."

NATURE OF THE RUBESCENS-HEMOLYSIN.

Like the hemolysin found in *Amanita phalloides*, and shown by Abel and Ford[1] to be a glucoside, the hemolysin in the *rubescens* is probably also a glucoside. By the methods already indicated[2] both uranyl acetate and freshly prepared metaphosphoric acid will remove the proteid from both concentrated and fresh extracts of the fungus, leaving the hemolysin unaltered in strength. After removal of the proteid, the solutions containing the still active hemolysins reduce Fehling's solution and ammoniacal silver nitrate directly, but have their reducing property increased by hydrolysis with dilute hydrochloric acid. The extracts ferment actively with brewer's yeast, pointing to the presence of free glucose. After treatment with both metaphosphoric acid and uranyl acetate, the actively hemolytic solutions, like those of the amanita-hemolysin, give no precipitate with phosphotungstic and phosphomolybdic acid, and the precipitate given by tannic acid is soluble in excess. An abundant precipitate is produced on the addition of basic lead acetate and also by cupric acetate. All tests for pentoses, α-naphthol and sulphuric acid, phloroglucin and hydrochloric acid, orcin, ferric chloride and hydrochloric acid have thus far given negative results. The *rubescens* hemolysin is therefore a glucoside, the exact composition of which is still under investigation. The important fact is that we have in this edible fungus powerful hemolysins but no Amanita toxin, the active principle of the "deadly Amanita."

AMANITA SOLITARIA.

This very rare fungus has an especial interest because it contains substances having a peculiar action upon blood corpuscles. It grows late in the summer, usually in the dry sandy soil just at the edge of

[1] *Loc. cit.*

[2] Abel and Ford, *loc. cit.*

the roads. It is a very large Amanita, pure white in color, developing from a universal cup like *Amanita caesaria*. In its early stages this cup forms an egg-shaped mass just projecting above the surface of the ground. As the plant matures the cup bursts and from its center the pileus issues, its surface covered with huge moist scales representing the remains of the cup. In the adult forms both pileus and stalk are covered with these scales, which give a fringed appearance to the entire plant. The scales are easily removed by brushing lightly with the fingers, but are quite viscid and sticky. The general structure of the plant resembles that of *Amanita phalloides*, but it is easily distinguished by its very large size and by its fringed surface. It is considered edible by most mycologists, by Atkinson and others, the danger from its use coming from mistakes in identification. No cases of poisoning due to it are on record.

Aqueous or saline extracts of *Amanita solitaria* are hemolytic, but not to such a degree as are other hemolytic Amanitas. The lysis is preceded by a typical agglutination of the corpuscles, which sink to the bottom of the tube in a densely adherent mass. The agglutination is slow, requiring one to three hours, and after this time a slow solution of the corpuscles takes place, requiring four to five hours. With concentrated extracts of perfectly fresh plants the reaction is more rapid, sometimes being complete at the end of two hours. The action upon corpuscles is thus seen to be closely analogous to that of an immune serum by which the erythrocytes are first agglutinated and then dissolved. When heated to 70° C., for half an hour, the hemolysin is destroyed, and filtered extracts heated to this temperature are innocuous to both rabbits and guinea-pigs. Even when introduced in concentrated solution only a transient edema is produced. Chemical analysis of the fungus confirms these observations. If extracts be evaporated to a small bulk *in vacuo* at 35° C., and then precipitated with ethyl alcohol, the precipitate contains the agglutino-hemolysin. The alcohol filtrate evaporated to dryness and taken up in water contains no Amanita toxin, and may be given to animals in large amounts without harmful results. Proteid may be removed from extracts of the "solitaria" by both the uranyl acetate and metaphosphoric acid methods, the proteid-free solutions retaining their power of agglutinating and dissolving the

erythrocytes. Uranyl acetate serves this purpose far better than does metaphosphoric acid, the latter destroying the hemolysin unless it be quickly neutralized by sodium bicarbonate. The proteid-free solutions reduce Fehling's solution and ammoniacal silver nitrate before boiling with hydrochloric acid, but have this property enhanced by this hydrolysis. No precipitate is given by phosphotungstic acid and the precipitate from tannic acid is soluble in excess. Precipitates are also given by basic lead acetate and by cupric acetate. Tests for pentoses with *a*-naphthol, orcin, and phloroglucin have thus far been negative.

The substances in *Amanita solitaria* acting upon blood corpuscles are thus seen to be glucosides, but differing markedly from those found in either *Amanita phalloides* or *Amanita rubescens*. It has not been possible to separate the agglutinating from the hemolytic action so that it cannot be stated whether more than one substance comes into play in this phenomenon or a single substance. As in the case of *Amanita rubescens*, the importance of the observation lies in the fact that an edible fungus may contain blood-laking principles but no Amanita toxin.

EXPERIMENTAL INVESTIGATIONS REGARDING THE ETIOLOGY OF DENGUE FEVER.*

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INTRODUCTION.

Having been instructed by the surgeon-general of the army to investigate the cause of dengue and to determine the possibility of the transmission of the disease by mosquitoes, we undertook the experiments detailed below when the opportunity was afforded by the occurrence of the disease in epidemic form at Fort William McKinley, Rizal, Luzon, P. I., four miles out of Manila. At this post, during the months of July, August, September, and October, there occurred over 800 cases of dengue, 128 of which were transferred to our dengue hospital in Manila for observation and experiment.

ETIOLOGY.

The etiological factor concerned in the causation of dengue has been sought for by almost every investigator who has studied the disease; thus, nearly every fluid, secretion, and excretion of the body has been examined, and, as might be expected, not a few observers have announced the discovery of the causative organism. Many of these so-called "discoveries" were made during the beginning of bacteriological science, and a perusal of the methods adopted in isolating the organisms described, as well as the description of their morphology, is sufficient to prove their absolute lack of scientific accuracy. We have not, therefore, deemed it necessary to review such contributions; but among the many who have investigated the etiology of this disease there are a few whose observations demand consideration. Among these may be mentioned McLaughlin, Graham, Carpenter and Sutton, Guiteras and Cartaya, and Agramonte.

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The early investigations regarding the etiology of dengue were directed toward a bacterial cause, but within the past few years the increasing importance of protozoa in the etiology of disease has turned the current of investigation, and almost all of the work that has been done upon this subject by recent investigators has been founded upon the belief that the disease is caused by some protozoon, probably by one infecting the blood.

The earliest attempt, based upon bacteriological methods, to discover the organism causing dengue was that of McLaughlin.[1] The descriptions of his experiments are detailed and the results obtained of interest, but, viewed in the light of the approved bacteriological methods of today, are open to very severe criticism and have not been confirmed by later observers.

McLaughlin examined both fresh and stained specimens of blood, made cultures from blood upon nutrient gelatin and also endeavored to secure cultures by partially filling sterilized glass bulbs with blood from a vein and incubating for weeks and even months. In the blood of every case upon microscopical examination he found micrococci, red or purplish in color, and in cultures upon nutrient gelatin, the same cocci, appearing black or brown when in masses, but when seen singly the red color was always distinct and characteristic. In one case, in the bulbs mentioned, which contained blood but no nutrient material, he found the same organism after an incubation period of from six weeks to three months.

While the researches of McLaughlin appear to have been partially accepted, or, at least, considered seriously, by some writers, we regard them as of purely historical interest, for reasons which are obvious.

The observations of Graham[2] of Beirut, published in 1903, renewed interest in the etiology of this disease, and a considerable amount of work has since been done with the object of confirming or disproving his results. He announced, as the result of his work, that dengue is caused by a protozoon, inhabiting the red-blood corpuscles, and closely resembling the plasmodia of malaria, except for the absence of pigment. He was not able to demonstrate the organism in stained specimens of blood, nor did he observe any sporulating forms. He considered that the organism underwent a developmental stage within the mosquito, and claimed to have demonstrated it in the cells of the salivary glands in mosquitoes of the genus *Culex fatigans,* but he was not able to observe any *zygotes* or any evidence of sexual forms within the mosquito.

Graham also details in his report the results of certain experiments which he made and in which he claims to have proven that dengue is transmitted by the mosquito. Of six healthy men bitten by infected mosquitoes (*Culex fatigans*) under circumstances which appear to have obviated all chance of infection in other ways, five developed dengue, two in four days, two in five days, and one in six days after having been bitten.

Because of the positive character of Graham's statements, his work attracted widespread attention, but though many experienced microscopists have endeavored to do so, none have been able to confirm his results as regards the presence of a parasite in the blood; but we believe that, however much he may have erred in his interpretation of the bodies described by him in the blood, his experiments regarding the method of transmission are most valuable, and his conclusion that dengue is transmitted by the mosquito is well founded and has been experimentally confirmed by us.

Upon the Isthmus of Panama, Carpenter and Sutton[3] studied 200 cases of dengue, examining both fresh and stained specimens of blood, but were unable to demonstrate either McLaughlin's micrococcus or Graham's protozoon. They also undertook some experiments with mosquitoes but did not obtain any results which they considered of importance.

Guiteras and Cartaya,[4] as the result of a very careful investigation carried on in Havana in 1905, believe that Graham is mistaken regarding his organism, and conclude, after examining a large series of blood specimens, taken during all days of the disease, at various hours, and stained by various methods, that the blood contains no structure resembling a parasite. They also endeavored to transmit the disease by mosquitoes, but with negative results. Guiteras states, regarding these experiments, that their small number and lack of variety deprive the negative result of a claim to conclusive character, and that their faith remains unshaken that the mosquito is the transmitter of dengue.

Agramonte,[5] studying the disease in Havana in 1906, was unable to demonstrate any parasite in the blood, nor was he able to transmit the disease by the mosquito. He states, however, that he believes that the mosquito transmits the disease and that his negative results were due to some undiscovered fault in technique.

The recent researches of Kieweit de Jonge and de Haan in Java and of Stitt[6] in Manila were without result as regards the presence in the blood of any parasite of etiological importance.

EXAMINATION OF BLOOD.

In attempting to solve the etiology of dengue and its method of transmission, our attention was first directed to the microscopical examination of the blood of patients suffering from the disease. Despite the failure of others to demonstrate any parasite in the blood, we considered that our work would be incomplete without careful examination of both fresh and stained preparations of the blood, and accordingly we have studied thoroughly, in this respect, a large number of our cases; the blood was examined during every period of the disease, but especially during the first two days and during the terminal rise in the temperature; various staining methods were used, including Wright's stain and the methods used in demonstrating *Treponema pallidum*. The latter methods were used very carefully and in numerous cases, as at the time we began our work we were greatly inclined to believe that the organism causing dengue might belong to the *Spirochetae*. We have not been able to confirm the results of McLaughlin or Graham, nor have we been able to demonstrate any organism in the blood of dengue patients, which we can consider as the cause of the disease.

The following is a résumé of the changes observed by us as occurring in the blood in dengue.

Hemoglobin and color index.—In uncomplicated cases the hemoglobin and color index are normal.

The red corpuscles.—Dengue is not a disease in which anemia is present. We have made numerous blood counts in severe cases, and have never observed a count lower than 4,500,000 red cells per c.m., even when the count was made at the termination of the disease. This fact alone appears to us to disprove conclusively the existence of Graham's hematozoon, which, by destroying the red corpuscles during its development within them, would certainly reduce them in number. We have never seen a case of uncomplicated dengue in which the clinical symptoms suggested anemia.

Morphology of the red corpuscles: In size the red corpuscles are unchanged. Poikilocytosis is not commonly observed, but in some cases, during the height of the fever, a moderate degree of poikilocytosis may be present. Crenation does not occur more rapidly nor is it more marked in dengue than in other acute febrile conditions. Vacuolation is common both in fresh and stained specimens of dengue blood, and in many instances the shape and appearance of the vacuoles is very suggestive of a parasitic invasion of the red cell; artefacts, due to degeneration of the protoplasm and clear areas due to the retraction of the hemoglobin, are common, especially in poorly prepared smears, and are well calculated to lead to error because of resemblance to bacterial or protozoal organisms. We have not observed that the appearance of the vacuoles occurring in the red corpuscles in dengue differs from that observed in many other febrile conditions, but it is certainly true that they frequently present an appearance very suggestive of ameboid motion without change of position; the progressive motion referred to by some writers we have observed in the case of rod-shaped artefacts, probably due to protoplasmic currents within the degenerating red cell.

It is not uncommon to observe in the blood of dengue, as well as in that of other febrile conditions, cocci or bacilli, either free in the blood plasma or attached to the red-blood corpuscles. In the vast majority of instances these bacteria are due to external contamination and have no relation to the disease in which they are observed. When they are attached to the red corpuscle, and still possess some motility, their resemblance to an intracellular parasite is often striking, but it is

usually possible, by gentle pressure, to dislodge them and thus demonstrate their real nature. We have not observed the presence of normoblasts or megaloblasts in the blood in dengue, and the absence, especially of normoblasts, indicates that anemia, even of a mild type, is not present.

The staining reactions of the red corpuscles in dengue do not differ from those present in health. Polychromatophilia or basophilia we have not observed, but in poorly prepared specimens the staining may be irregular, suggesting granular degeneration.

The leucocytes.—Number: One of the most important blood changes in this disease is the presence, in almost every case, of a marked leucopenia. From our observations we are convinced that the leucopenia of dengue is almost constant throughout the attack, and that it is of considerable diagnostic importance. We have made leucocyte counts in a large number of cases and have invariably found marked reduction in the total number of leucocytes, with, as will be seen later, quite a characteristic change in the relative proportion of the various forms. The lowest leucocyte count we have seen was 1,200 per c.m., the highest, 4,860 per c.m. the average, 3,800 per c.m. We have found that the leucopenia is progressive, being most marked upon the fifth day of the disease, or, sometimes, upon the sixth.

Morphology: We have observed no morphological changes in the leucocytes, or any evidence of the presence of a leucocytozoon.

Differential leucocyte count: From the studies of Carpenter and Sutton, and later, of Stitt, the differential leucocyte count in dengue has assumed considerable diagnostic importance, and, taken together with the leucopenia, appears to us to be entitled to very careful consideration in the differential diagnosis of dengue, yellow fever, malaria, and the eruptive fevers. Carpenter and Sutton,[7] from their blood examinations, conclude that in dengue there is always a leucopenia, and generally an increase in the small lymphocytes and in the eosinophiles, the latter occurring late in the disease. Stitt[8] made differential leucocyte counts at varying periods of the disease. He found marked variation occurring in the number of the various forms of leucocytes at different periods, there being at first a great increase in the small lymphocytes, succeeded by a greater increase in the large

ymphocytes and mononuclears, and finally, during the terminal eruption, a most marked increase in the large mononuclears.

Because of lack of time we have made but comparatively few differential counts, but our results have been supplemented by those of Lieutenent Vedder, Medical Department, U. S. A., stationed at Fort William McKinley, who kindly volunteered to assist us in this direction. From our own observations we are loth to lay as much stress upon the variation of the relative proportions of the large and small lymphocytes, at varying stages of the disease, as does Stitt, for in many instances we have found that no constant relationship exists between the variety of lymphocyte increased and the period of the disease, but we have found a constant leucopenia, a decrease in the polymorphonuclears, and an increase in small lymphocytes at every stage. In one of our experimental cases in whom we produced a severe attack of dengue by the intravenous inoculation of filtered blood from another experimental case, the leucocyte count, made upon the first, third, and sixth day of the disease, well illustrates the changes described by Stitt, as is shown by the following record of the counts.

FIRST DAY OF DISEASE.

Polymorphonuclears	50.0%
Small lymphocytes	41.0
Large lymphocytes	7.5
Eosinophiles	1.5

THIRD DAY OF DISEASE.

Polymorphonuclears	52%
Small lymphocytes	36
Large lymphocytes	8
Eosinophiles	4

SIXTH DAY OF DISEASE.

Polymorphonuclears	48%
Small lymphocytes	14
Large lymphocytes	32
Eosinophiles	6

It will be observed that the eosinophiles increased as the disease progressed, and this has been noticed in many of our cases. While the above differential count is typical of the results obtained by some observers, we have found, even in a limited number of examinations, that it is not of sufficiently frequent occurrence to be depended upon in

reaching a diagnosis. In fact, in most of our counts we found that the small lymphocytes outnumbered the large in every stage of the disease.

Vedder, who made hundreds of blood counts upon patients suffering from the disease in the same epidemic as that from which we obtained our material, found that the polymorphonuclear leucocytes were greatly decreased, and the small lymphocytes greatly increased during every stage of the disease. He also found that the large lymphocytes are moderately increased during the latter days of the illness. His results will be published later in full.

Blood plates.—We have observed no changes in the number or appearance of the blood plates in dengue.

Blood plasma.—In neither fresh nor stained specimens of dengue blood have we been able to demonstrate any organism of etiological significance in the blood plasma. In a few instances bacteria were noticed, but from the ease with which blood cultures become contaminated in this climate, we believe that these bacteria were of external origin. The most common bacterium observed was a long, stout bacillus, actively motile, and commonly seen here in blood specimens from various sources. No organism resembling a protozoon was observed in the blood plasma. Yeast cells were frequent contaminations in stained specimens.

Summary.—From our examinations of the blood in dengue we consider that the following conclusions are justified:

1. There does not occur in the blood of dengue any visible organism, either bacterial or protozoal in nature, which can be considered as the cause of the disease.

2. Dengue is not accompanied by anemia, the red blood count being normal in uncomplicated cases. There are no characteristic morphological changes in the red corpuscles, leucocytes, blood plates, or blood plasma.

3. Dengue is characterized by a leucopenia, and, in the vast majority of instances, by a decrease in the polymorphonuclear leucocytes and a marked increase in the small lymphocytes; the increase in the small lymphocytes is constant throughout the disease.

BLOOD CULTURES.

With the exception of McLaughlin's researches which have been mentioned, and which, so far as culture methods are concerned,

were almost valueless, we have not been able to find in the literature any detailed descriptions of experiments having for their object the cultivation of bacteria or protozoa from the blood of dengue patients. In view of the success attained by Novy and others in the cultivation of trypanosomes, and by Rodgers, in cultivating the Leischman-Donovan body (*Herpetomonas donovani*), we were especially hopeful, that by applying similar methods in this disease, we might be able to secure growths of any protozoon which might be present. As we have stated, we were impressed with the idea that dengue might be caused by an organism belonging to the same group as those causing the relapsing fevers, or to some closely allied group, and we therefore endeavored, by employing special staining methods and culture media, to demonstrate such an organism.

Methods.—In our experiments we have used citrated blood and acid and alkaline broth as culture media. In making cultures with citrated blood, the sterilized syringe was first filled with citrate solution, which was then ejected, a very little being allowed to remain in the needle; the syringe was then filled with blood by plunging the needle into a prominent vein in the forearm of the patient and withdrawing the blood very slowly until the barrel of the syringe was full; the blood was then ejected into small sterilized glass tubes and kept at room temperature and in the lower compartment of the ice-box, the latter in order to give any organism undergoing a portion of its life cycle in a cold-blooded animal surroundings congenial to its development.

In making blood cultures in broth, 10 c.c. of blood, obtained from a vein of the forearm, were added to 250 c.c. of the bouillon, contained in 500 c.c. flasks, and incubated at temperatures of from 80° to 98° F.

Citrated blood cultures.—In eight cases we endeavored to secure cultures of the organism causing dengue by citrating blood from dengue patients, obtained at various periods of the disease. In none of the cases have we been able to demonstrate any organism in the culture which we can consider as of etiological significance; in none of the tubes of citrated blood did we observe any organism resembling, in the least, a protozoon, and all of the bacteria observed were evidently external contaminations; one, a small diplococcus, occurred in two of our cultures, but in the light of our later work with filtered blood, was evidently of no importance.

Broth cultures.—In twelve cases we used broth blood cultures, allowing them to incubate for as long as eight weeks. The majority of the flasks became contaminated, but in four case the blood cultures

did not show any growth at the end of eight weeks, when they were destroyed. A staphylococcus grew in one at the end of 48 hours; a diplococcus in three in 72 hours, accompanied by a large spore-bearing bacillus in two of the cases; a short, thick, motile bacillus together with a staphylococcus in one in four days, and various spore-bearing bacilli in the remainder. These organisms we regarded as contaminations and therefore did not experiment with them, the result of our work with filtered blood later confirming our opinion.

Summary.—As the result of our culture experiments we were forced to conclude that no organism was found constantly enough in our cultures to warrant us in regarding it as the cause of dengue, especially as a number of cultures remained sterile, though kept for as long as eight weeks. We observed no organism in any of our cultures which in the least resembled a protozoon.

INOCULATION OF DENGUE BLOOD.

Having thus failed to demonstrate any organism in either fresh or stained specimens of blood from dengue patients, or in our blood cultures, we directed our attention to the possibility of producing the disease in healthy men by the inoculation of blood from those suffering from dengue; fortunately for the success of our work, we were dealing with a disease which in the young and vigorous is not dangerous to life, and for this reason we felt justified in making the experiments. We hoped in this way to determine the presence or absence of the infective agent in the blood, for should such experiments prove successful they would demonstrate that the cause is in the blood, and that insect transmission, therefore is possible, whereas negative results would prove that the blood did not contain the organism unless it be one that first has to undergo a developmental cycle outside the body before it can infect man.

In order to secure subjects for experiment, a call for volunteers was issued to members of the Hospital Corps, U. S. A., serving at the Division Hospital, Manila, and as a result, four men volunteered, in all of whom we succeeded in producing dengue by intravenous inoculation of blood from cases of the disease. We desire to express our admiration of the courage and devotion to duty of these men, who, with no prospect of pecuniary reward, cheerfully placed themselves in our hands for these experiments.

As more men were needed, and as no more Hospital Corps men were available, we consulted Major-General Leonard Wood, commanding the Philippines Division, who authorized us to offer a reward for volunteering, as a result of which we secured more men than we could use, as we were limited to 16, including those already experimented upon. Unfortunately, of the 14 men* we have experimented upon to date, seven came from Fort William McKinley, having passed unharmed through the extensive epidemic of dengue at that post, and of these men we found two absolutely immune, three relatively immune, and one doubtful. Of the same number of Hospital Corps men who had not been exposed to dengue we found only one immune.

1. *Intravenous inoculation of unfiltered dengue blood.*—Eleven of our 14 volunteers were given intravenous inoculations of unfiltered dengue blood, and of these, seven developed the disease, while in one case the result was doubtful. In three of the cases there existed an absolute immunity to the disease, as proven by our experiments.

Experiment 1.

Case 1.—Chart 1. E. W. Private. Hospital Corps, U. S. A. Had not been exposed to dengue. At 3:30 P. M., July 24, 1906, he was given an intravenous injection of 20 minims of unfiltered dengue blood from Case 20 (see Chart A). The patient from whom the blood was taken for the inoculation had a mild attack of dengue and was probably nearly over the disease at this time. We believe that this accounts for the mild character of the experimental disease in this case, for while the symptoms present were typical, it will be seen upon referring to the temperature chart that the fever was slight as compared with our other experimental cases. The following is a résumé of the clinical record in this case.

July	24.	At 3:30 P. M., inoculated intravenously with 20 minims of blood from Case 20. Subject in good health and temperature normal.
"	25, 26, and 27.	Patient feeling well.
"	28.	Has some fever and headache. Bowels constipated.
"	29.	Patient feels uncomfortable, complaining of vague muscular pains and smarting of the eyes; eats and sleeps fairly well. Bowels regular.
"	30.	Last night had severe headache, pain in eyes, in lumbar muscles, and in the elbows, ankles, and wrists. At present (11 A. M.) complains of dull headache and slight lumbar pain; the eyes are painful, the pain being aggravated by movement of the eyeballs. Tongue moist and clean.
"	31.	Feels much better, the pain in the head and the muscles having disappeared. A slight eruption is present upon the back and chest.
August	2.	Fells well. Eruption has disappeared.
"	6.	Returned to duty.

*Of the fourteen soldiers who volunteered for this work, seven belonged to the Hospital Corps, U. S. A.; three to the Eighth U. S. Cavalry; two to the Sixteenth U. S. Infantry; one to the Thirteenth U. S. Infantry, and one to Company B, Engineer Corps, U. S. A.

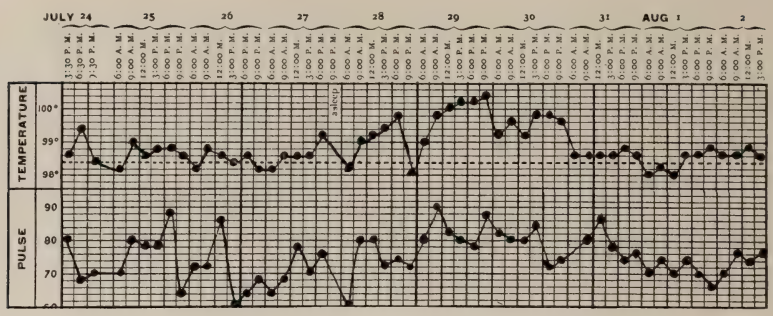


CHART 1.

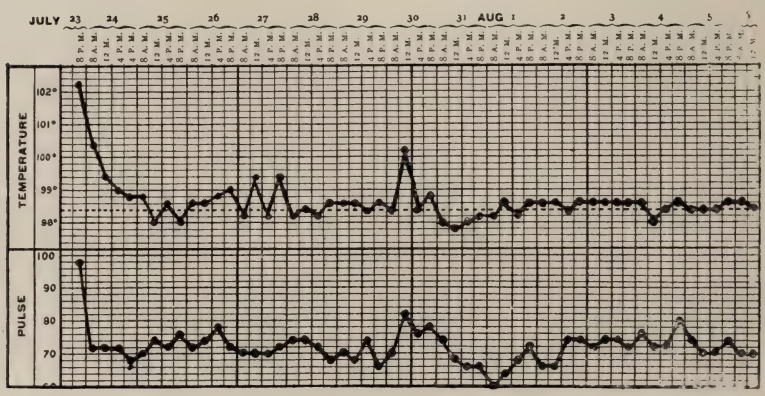


CHART A.

Remarks.—Upon reference to the temperature chart (Chart 1) it will be observed that the temperature began to ascend about 9 A. M. on July 28, but the patient complained of no symptoms until nearly 24 hours later. The incubation period in this case is, therefore, about 3 days and 18 hours, reckoning it from the morning of July 28. The eruption present in this case was a typical dengue eruption but was not very marked, only appearing over the chest and back.

Experiment 2.

Case 2.—Chart No. 2. W. R. H. Private, first class, Hospital Corps. Not previously exposed to dengue and in good health. At eleven o'clock, upon the morning of July 31, 1906, he was given an intravenous inoculation of 20 minims of blood from Case 30 (Chart B), who was suffering from a typical attack of dengue of four days' duration at the time the blood was taken. No symptoms appeared in Case 2 until early in the morning of August 3, as is shown by the following résumé of his clinical record:

July 31. Inoculated with dengue blood as stated.
August 1. and 2. Feels well.
" 3. At 2 A. M. this morning was awakened by pain in the chest and difficulty in breathing. This soon passed away and patient slept until morning. Felt well upon awaking this morning, but soon developed sharp pain in the head, and in the muscles of the back and legs. Complains also of a slight cough and pain in the eyes; had a slight chill at 5:30 P. M. today. No appetite.
" 4. Patient complains of severe pains in the lumbar region but has no headache. Diarrhea is present, the stool being watery in character. No appetite. His general appearance is typical of dengue.
" 5. Still complains of the lumbar pain; at 9 P. M. complained of abdominal pain, nausea, and vomited once. No eruption has been observed.
" 6 and 7. Patient feeling well.
" 8. Has severe headache and pain in the muscles and joints. Last night was very nervous, almost delirious.
" 9. Feeling well. Returned to duty August 14, 1906.

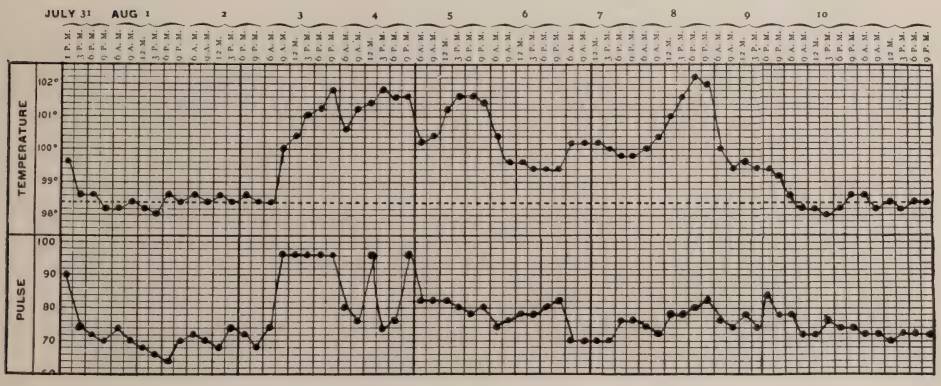


CHART 2.

Remarks.—In this case the incubation period, as shown by the temperature chart, was approximately 2 days and 19 hours. The temperature curve is typical of a moderately severe case of dengue fever and the symptoms corresponded; the terminal rise and fall is well shown in this chart. No eruption occurred at any time, although otherwise the symptoms were typical, with the exception of the diarrhea, which was present for a short time, accompanied by nausea and vomiting; these symptoms we regard as rather the exception than the rule in dengue, and we are inclined to believe that in this case they may have been excited by the presence in another tent of a case of cholera, a disease which just at this time was common in Manila.

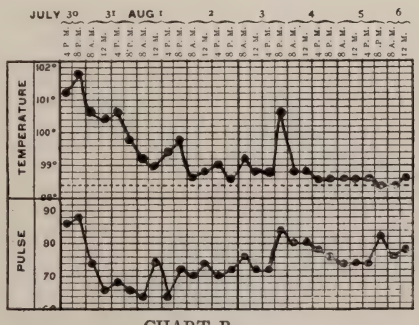


CHART B.

Experiment 3.

Case 3.—Chart 3. E. W. Private, first class, Hospital Corps, U. S. Army. At the time of inoculation this man was perfectly well and had not been exposed to dengue. He was given an intravenous inoculation of 20 minims of dengue blood at 2 P. M., August 16, 1906, from Case 36 (see Chart C). The latter case was a most typical one of dengue, which, at the time the blood was taken, had lasted a little over three days. The clinical record of Case 3 follows:

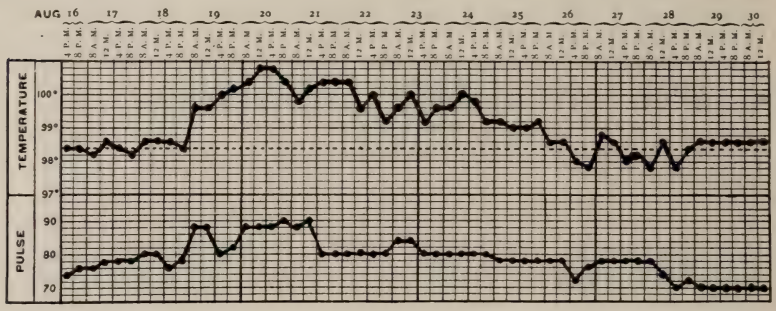


CHART 3.

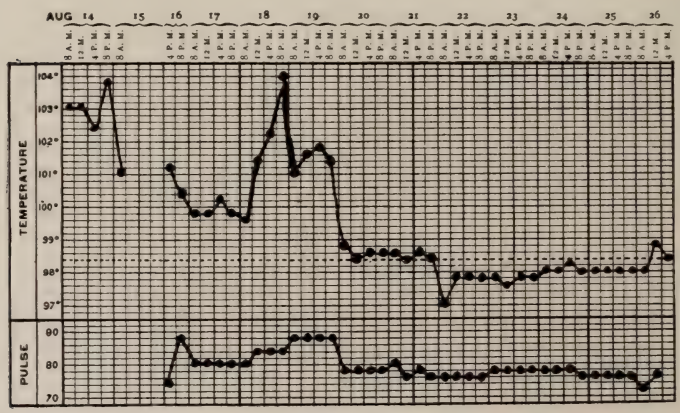


CHART C.

August 16. Inoculated with dengue blood as stated.
" 17. and 18. Feeling well.
" 19. Slight temperature last night, but no headache, pain or other symptoms.
" 20. Last night suffered from headache, pain in the bones and muscles of the back, and severe pain in the knee joints. This morning has headache located in the temporal region, and general muscular pain. Eyeballs pain and are tender to the touch. Stomach and bowels normal. An eruption appeared this morning, and covers almost the entire body but is especially marked over the trunk and arms; it consists of very fine, slightly elevated areas, dark red in color, the color disappearing upon pressure.

August 21. Feels better this morning, but still has pain in eyes, shoulders, knees, and wrists. The eruption is still present but is not as marked as yesterday. Appetite poor. Bowels regular. Tongue moist and clean.

" 22. Patient had more headache last night but feels much better this morning. The eruption is still present and itches greatly. The bowels are loose. Appetite good.

" 23. Had headache last night which caused insomnia, also aching pains, severe in character, in the muscles and joints. The eruption has greatly increased covering the entire body, being especially marked over the trunk and limbs. It is a typical dengue eruption.

" 25. Patient slept well and all symptoms have disappeared. The eruption is fading and the itching is very annoying; there is considerable desquamation in the form of fine white scales.

" 27. Eruption has disappeared. Patient is well and upon August 31, 1906, was returned to duty.

Remarks.—The incubation period in this case was about 2 days and 18 hours. The temperature curve is not as characteristic as is generally observed, but the symptoms were very typical, and the fact that we were able to produce a very severe case of dengue by the injection of the filtered blood of this patient proves beyond doubt the nature of the disease (see Case 9, Chart 9). An interesting feature of this case was the early appearance of a well-marked eruption, which, after fading gradually, increased again during the crisis and finally disappeared, accompanied by considerable desquamation. It is also interesting to note that in the case of dengue referred to as being produced by the injection of filtered blood from this case, the eruption appeared early and presented the same characteristics. While the temperature was low the symptoms in this case were more severe than in many others in which the fever was much greater, and the eruption was almost as well marked as in any case that we have observed.

In the three cases just described the inoculations were made as soon as the subject volunteered, no previous experiments in the way of exposure to fomites or mosquitoes having been tried. In the cases which follow the inoculations were used as a final test of immunity, the men inoculated having been exposed both to fomites and supposedly infected mosquitoes.

Experiment 4.

Case 4.—Chart 4. C. H. B. Private, Troop A, Eighth U. S. Cavalry. Had been exposed to dengue, his troop having had 18 men upon sick report with it. At time of volunteering was in good health, and stated that he had never had any serious illness. The following is a résumé of the clinical record in this case.

September 22. Exposed to fomites of dengue, being placed with three dengue cases in a mosquito-proof tent, sleeping in their beds and wearing their underclothes.

" 26. No results being obtained, the patient was placed under a mosquito bar with mosquitoes that had bitten Case 38, a typical case of dengue, the night before.

" 27 to Oct. 2. During this time the subject has slept under the mosquito-bar containing the infected mosquitoes, but claims that he has not been bitten at all and his statement is confirmed by the fact that the mosquitoes all remained empty during this time, and made no attempt to bite, most of them dying while in the mosquito bar. He states that so far as he knows he has never been bitten by a mosquito, although he has campaigned in localities in Africa which were almost uninhabitable because of these insects. At first we were inclined to doubt his statements in this respect but observation has convinced us that this man is really immune to mosquitoes.

October 3. At 10 A. M. the subject was given intravenously 20 minims of unfiltered blood from Case 44, a very typical case of dengue of about $3\frac{1}{2}$ days duration.

" 4 and 5. Feeling well.

" 6. Last night, about 10:30 P. M., the patient complained of fever and muscular pains. This morning has severe pain in the head, back, and limbs. Face greatly flushed, conjunctivae congested. Bowels constipated. Tongue moist with thin white coating.

" 7. Vomited last night. Has less pain this morning, located mostly in the muscles of the loins and thighs. Has much headache and pain in the eyeballs upon moving them.

" 9. Feeling well.

" 10. Had headache and muscular pains last night. Feels better this morning.

" 15. Returned to duty.

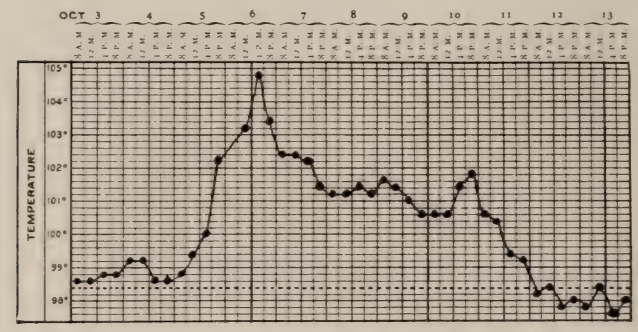


CHART 4.

Remarks.—The temperature curve in this case is one often observed in naturally acquired dengue, and the symptoms throughout were typical of the disease. The absence of an eruption is to be noted; not that it is unusual in many natural infections, but because in our experimental cases an eruption was almost always present. Thus of the 10 cases in which we were successful beyond doubt in producing dengue, eight presented well marked eruptions, while one was somewhat doubtful in this respect.

Experiment 5.

Case 5.—Chart 5. C. R. D. Second-class private, Co. B, Engineer Corps. U. S. Army. This man at the time of volunteering was in good health, but had been exposed to dengue during the Fort McKinley epidemic. He was observed for a period of four days before any experiments were made. The following is the clinical record of this case.

September 19. At 1 P. M. a small abrasion was made upon the mucous membrane of his cheek, and he then rinsed his mouth with diluted blood from a dengue case. No results were obtained from this experiment.

October 3. Has been exposed to fomites as described in Case 4, since September 22. No results obtained.

" 4. Patient slept last night under mosquito-bar containing mosquitoes that had bitten a typical case of dengue the night before. He was bitten several times during the next few nights but dengue did not develop.

" 22. At 3 P. M. today an intravenous injection of 20 minims of blood from a dengue case, Case 60, was given the subject.

October 23, 24, and 25. Feeling well.
" 26. At 7 P. M. today the patient complained of headache, pain in the lumbar region and in the legs, and loss of appetite.
" 27 and 28. Patient complained of severe headache, pain in the lumbar region and in the joints. His hands and wrists are swollen slightly, and his face, arms, and hands greatly flushed. He is constipated and has no appetite.
" 29. Feeling much better. An eruption has appeared upon the chest and abdomen, resembling more the eruption due to heat than a dengue eruption.
" 30 and 31. Patient feels well. There is a well-marked rash over the back and chest which upon the 31st had extended over the arms and legs. This rash is a typical dengue rash.
November 1. The rash is still well marked and is present over the entire body, including the palms of the hands and the soles of the feet.
" 2. Patient feeling well, and eruption has almost disappeared.
" 5. Returned to duty.

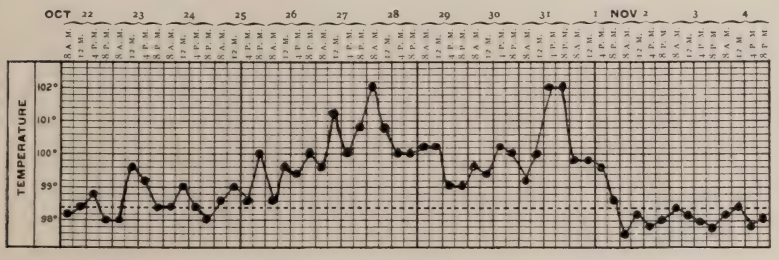


CHART 5.

Remarks.—It will be observed that this man did not contract dengue through the mucous membrane of the mouth, nor from mosquito bites. As regards the latter experiment, we cannot be sure that he was bitten more than one or two times by the mosquitoes, as they disappeared very rapidly from the bar in which they were confined. From the intravenous inoculation of unfiltered dengue blood he developed a very typical attack of the disease, as will be seen by referring to the temperature chart. The incubation period in this case was longer than in any of our previous cases, being four days and four hours.

Experiment 6.

Case 6.—Chart 6. J. E. S. Private, Troop H, Eighth Cavalry. At thetime of volunteering this man was in good health but had been exposed to dengue at Fort McKinley. The following is a record of the experiments performed in this case.

September 22. Exposed last night to mosquitoes that bit Case 41 on September 13. Says that none bit him during the night. September 23: exposed again last night to same mosquitoes and says he was bitten once. Dengue did not develop.
October 7. Was exposed to mosquitoes that bit Case 44 the night before.
" 8 to 15. Feeling well, and states that he does not know whether he has been bitten. Dengue did not result.
" 25. Exposed to mosquitoes that had bitten Case 60 the night before. Was bitten at least twice. Mosquitoes had disappeared from the bar by October 28, and he was not bitten again. Dengue did not result from this experiment.

October 31. Exposed to mosquitoes that had bitten Case 65 the night before. Dengue did not result.

November 8. At 3 P. M. today the patient was inoculated intravenously with 20 minims of dengue blood from Case 70. The man inoculated from had a typical attack of dengue fever, and the inoculation was made upon the third day of the disease. (Temperature 101.4°.)

" 10 to 14. Subject feeling well.

" 15. In the afternoon the subject had a slight chill accompanied by pain in the muscles and severe headache.

" Patient complains of severe headache, backache, and pain in the limbs.

" 17 to 20. During this time the patient presented the usual symptoms of dengue, which have already been indicated.

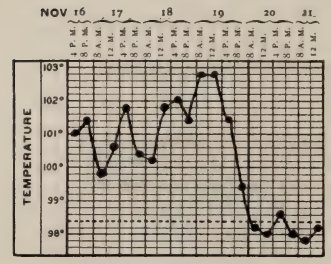


CHART 6.

Remarks.—The chief point of interest in this case is the long period of incubation, exceeding that of any experimental case that we have observed. Inoculation was made as 3 P. M., November 8, and the first symptoms appeared upon the afternoon of November 15, making the incubation period about seven days. It should be remembered that this man had already passed unharmed through a severe epidemic of dengue, and it is probable that he possessed a relative immunity to the disease, although his clinical symptoms were typical and rather severe in character. He developed a typical dengue eruption just before the crisis, which disappeared with the fever.

Experiment 7.

Case 7.—Chart 7. W. J. Private, Troop D, Eighth U. S. Cavalry. This case is of interest because the experimental dengue was complicated by an attack of malarial fever. The man had been exposed to dengue at Fort McKinley, but was in good health at the time of volunteering. The following is the clinical record of his case:

September 19 to 26. Exposed to fomites of dengue during this time in the manner described.

" 26. Exposed to mosquitoes that had bitten Case 80 (Chart L) the night before. This case was afterward found to be suffering from aestivo-autumnal malarial fever.

" 27 to 29. Subject feeling well.

October 8. Exposed to mosquitoes that had bitten Case 4 (Chart 4) the night before, and was bitten twice upon the night of October 9. Dengue did not result from this experiment.

" 25. Exposed last night to mosquitoes that had bitten Case 81 (typical dengue) the night before. States that he did not feel well during the night, and complains of headache.

" 26 to 28. Aestivo-autumnal parasites were found in his blood on the afternoon of October 27, and quinine was at once administered. Feels well upon the 28th.

November 17. Inoculated at 10:30 A. M., with 20 minims of blood from Case 82, who was suffering from a typical attack of dengue which had lasted about three days.

" 18 to November 23. Feeling well until November 31, when he says that he developed slight muscular pains which have persisted since.

" 24. At 10 A. M. patient had a slight chill followed by a high fever. Upon November 22 an eruption had been noticed covering the entire body, which resembled the eruption of dengue and which is still present.

November 26. Patient is feeling well and is free from pain. He states that he had had considerable pain before his chill for several days. He is covered with an abundant rash, which presents all the characteristics of that observed in our other dengue cases.

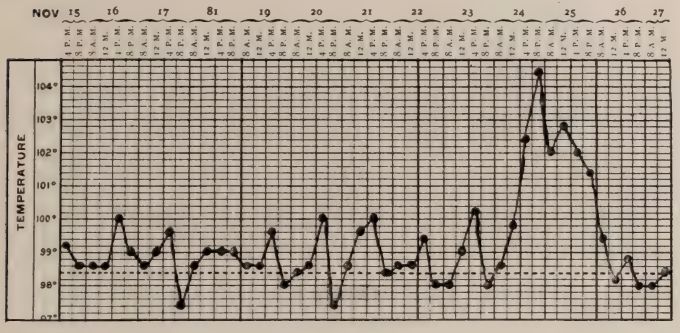


CHART 7.

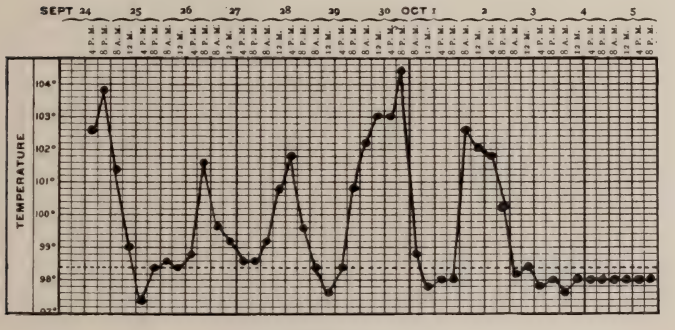


CHART L.

Remarks.—It is difficult in this case to determine the exact period of incubation, and the chart is also atypical because of the concurrent malarial infection. The clinical symptoms, however, were typical, and the presence of the rash removes all doubt as to the nature of the infection.

Experiment 8.

Case 8.—Chart 8. R. R. Private, Co. H, thirteenth U. S. Infantry. This man was in good health at the time of volunteering, but had been exposed to dengue during the epidemic at Fort McKinley. The following is the clinical record of this case:

September 12. Inoculated intravenously with one-half minim of blood from Case 83 (Chart O), who was suffering from a typical attack of dengue.

" 15. Patient states that he had a severe headache last night. Still complains of headache and pain in the arms and legs.

" 16. Still complains of headache, but has no other pain. Bowels constipated. Appetite poor.

" 17 to 19. Patient feeling well.

September 19. Inoculated intravenously at 1 P. M. with 1 c.c. of filtered blood from Case 2 (Chart 2). No result.

" 25. Inoculated intravenously at 1 P. M. with 20 minims of blood from Case 38. No result.

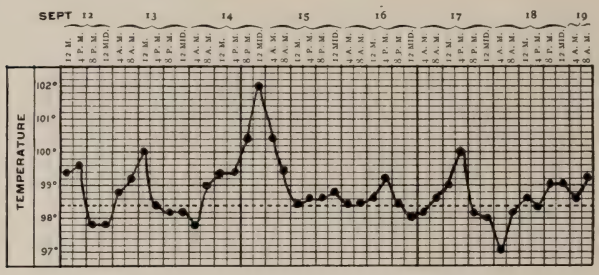


CHART 8.

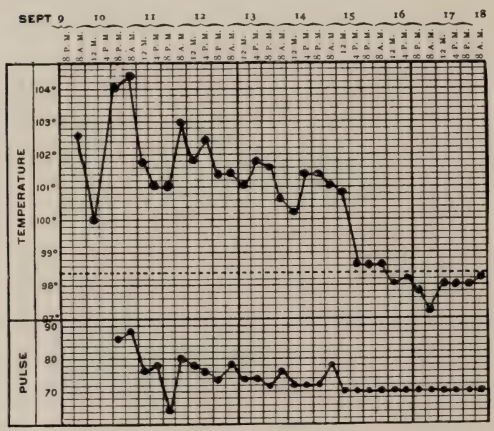


CHART O.

Remarks.—We have regarded this case as doubtful, although we are inclined to believe that the rise in temperature upon September 15 was due to a slight attack of dengue produced by the inoculation of the one-half minim of dengue blood. This is much less blood than we have used in our other experiments, and it may be that the slight symptoms produced may be due to this fact. If this man did not suffer from an attack of dengue as the result of his first inoculation, he must have been immune, as neither the subcutaneous injection of the filtered dengue blood or the intravenous injection of unfiltered dengue blood produced the disease.

Summary.—The intravenous injection of unfiltered dengue blood into healthy men is capable of producing a typical attack of dengue in such men. Thus of 11 men so inoculated, seven suffered from dengue fever, while in one the result was somewhat doubtful. Three of the men were absolutely immune to the disease.

2. *Intravenous inoculation of filtered dengue blood.*—Having proven by our inoculation experiments with unfiltered blood from dengue patients that the disease could be thus transmitted, and, furthermore, that while the cause must be present in the blood, it is not possible to demonstrate it in either fresh or stained specimens, or in cultures, by any known method of examination, we were forced to the conclusion that the causative organism must be ultramicroscopic in size, as in the case of yellow fever, rinderpest, hog cholera, South African horse sickness, etc. In order to determine if dengue belonged to this class of infections, we determined to try the effect of the intravenous inoculation of filtered blood from dengue patients into healthy men. We have experimented in this way upon two men, in both of whom we have been successful in producing very typical attacks of dengue accompanied by rather severe symptoms.

Filter used and control methods.—In our filtration experiments we have employed a Lilliput filter made of diatomaceous earth, which was carefully tested each time that it was used. Before using, the filter was sterilized and the filtration done under pressure produced by a water vacuum. We are unable to state the exact amount of pressure obtained in this way but the vacuum was low and the pressure used could not have exceeded one atmosphere.

After filtering the blood the following control test of the filter was made in each case: A suspension, in nutrient broth, was made of *M. melitensis* and *Sp. cholerae*, and this filtered through the filter used in filtering the blood; the filtrate was then incubated for two weeks, daily examinations of it being made during this time.

The filter we used retained both of these organisms, the filtrate remaining sterile for two weeks, when it was thrown away. Besides the control test of the filter, we kept, in each instance, a portion of the filtered dengue blood for a period of 10 days, making daily examinations, and in one case, we made several cultures from the filtered blood in broth, but no growth was obtained in either the filtered blood or the cultures.

Experiment 9.

Case 9.—Chart 9. E. J. D. Private, Hospital Corps, U. S. A. Upon August 21, 1906, 10 c.c. of blood were drawn from the median basilic vein of Case 2, an experimental case of dengue which has been described (see Case 2, Chart 2). The blood

was taken upon the third day of the disease, the symptoms of the patient at the time consisting of fever, headache, severe pain in the muscles of the shoulders and in the wrists and knees, while there was present a typical dengue eruption.

The blood was rapidly defibrinated, diluted with an equal amount of salt solution, and filtered through a Lilliput filter, controlled as described. The filtration of a sufficient quantity for use was completed in about three-quarters of an hour. Of the filtrate, 50 minims, containing 20 minims of the filtered blood, was inoculated intravenously into Case 9 at 4.20 P. M., August 21. The patient at the time of inoculation had been in the hospital under observation for several weeks, and as no cases of dengue had occurred in the hospital during that time, he had not been exposed to the disease. No symptoms of importance developed until August 25, the period of incubation being 3 days and 11 hours. Previous to the decided onset of the disease there had been slight fever and some pain in the back, symptoms which were probably due to a chronic gonorrhea from which he had suffered for some time. The following is the clinical record of this case:

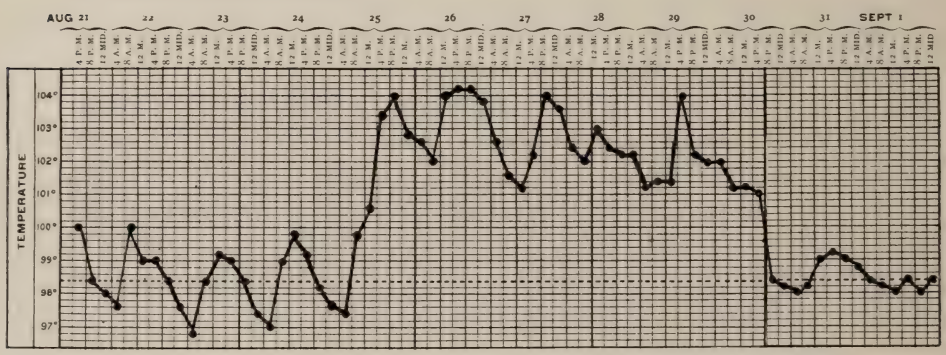


CHART 9.

August 21. Inoculated intravenously at 4:20 P. M. with 20 minims of filtered blood from Case 2.

" 22 and 23. Feeling well.

" 24. Slight muscular pains.

" 25. 9 A. M. Complains of pain in muscles of neck, shoulders, and knees. Has some headache; bowels constipated. Tongue moist and clean. 4 P. M. The symptoms have increased in severity. The headache is intense; there is severe pain located behind the eyeballs which are painful on pressure. There is general muscular pain, especially in the muscles of the jaw, lumbar region, and in the calves of the legs. Patient states that his bones ache, and that he is unable to rest comfortably in any position. He also complains of severe pains in the articulations. An eruption is present, covering the chest, abdomen, and thighs, being especially marked over the forearms and around the wrists; it is dull red in color, consisting of minute elevations surrounded by a vivid flush, which makes the rash appear confluent.

" 26. Patient passed a very restless night, suffering from insomnia and severe pain in the back, chest, legs, head, and eyes. This morning still has severe pain in these regions. There has been no vomiting and the bowels are constipated. Tongue is moist with a white coating. The eruption covers the entire body, and is more marked than yesterday. There is complete loss of appetite, and the patient is very restless.

August 27. Feels much more comfortable this morning. The steady ache in the muscles has disappeared but he still suffers from lancinating pains in the head, back, and legs. The eruption has almost disappeared.
" 28. Passed another restless night, and suffered a great deal from pain in the muscles. This morning he complains of severe headache and pain in the loins and legs. The eruption has faded from the trunk.
" 29. Feels better this morning. Still has headache and pain in the eyes, but the general muscular pain has disappeared.
" 30. Still complains of pain in the head and eyes, but slept well last night.
" 31. Patient states that he feels very well this morning. Has no pain, and appetite is returning. There is present a very profuse dengue eruption covering the entire body, especially marked upon the arms, legs, hands, and feet.
September 1. Feels well. The eruption is less distinct, although it still covers the entire body.
" 2. Is feeling well in every way, and the eruption has disappeared.
" 4. Discharged from our experimental ward.

Remarks.—This case, as shown by the clinical record and the chart, is typical of severe dengue, but the initial eruption was more marked than in any of our cases. The patient suffered greatly from headache and general muscular pains, and repeatedly stated that he felt as though every bone in his body had been broken. The temperature chart presents a high range of fever, with not as marked a period of remission as is generally observed; it will be noted that morning remissions occurred regularly, but that in the afternoon the temperature ascended, reaching 104° F. on three successive days. A more permanent remission occurred upon the fifth day, succeeded upon the sixth by the final rise and the crisis, the temperature reaching normal upon the seventh day of the disease.

An eruption appeared in this case upon the second day of the disease (the so-called initial eruption) extending over the chest, abdomen, and thighs. The typical dengue eruption occurred, as is usual, during the crisis, and was very profuse, extending over the entire body, even the hands and feet being covered with it. The severe initial eruption in this case is very unusual, and it is most interesting to find, upon reference to the clinical history of Case 2, from whom this man was inoculated, that an eruption occurred in that case also upon the second day of the disease.

Experiment 10.

Case 10.—Chart 10. B. S. First-class private, Hospital Corps, U. S. Army. Upon August 31, 1906, at 12:15 P. M., this man, who had been on duty at the Division Hospital for weeks, had been carefully observed, and had not been exposed to dengue, was given an intravenous injection in the arm of $3\frac{3}{4}$ c.c. of normal salt solution containing 20 minims of filtered dengue blood from case No. 87 (Chart H). Ten cubic c.c. of blood was taken from the median basilic vein of Case 87, at 10:30 A. M., August 31, diluted with normal salt solution and filtered through the same filter used in Case 9, the filter being controlled as has been described. This filtered blood was used for the inoculation. The patient from whom the blood was obtained was suffering from a rather severe attack of dengue and the blood was taken upon the fourth day of the disease.

After inoculation with the filtered blood no symptoms appeared in Case 10 until midnight of September 3, but upon referring to the temperature chart it will be noticed that fever had been present for at least sixteen hours before it was complained of. If we assume that the first rise in temperature indicates the onset of dengue in this case,

the incubation period must have been about 2 days and 12 hours, while if the first symptom complained of by the patient be considered as marking the onset, the incubation period would be three days. We consider that the incubation period in this case was $2\frac{1}{2}$ days. The following is a résumé of the clinical record of this case:

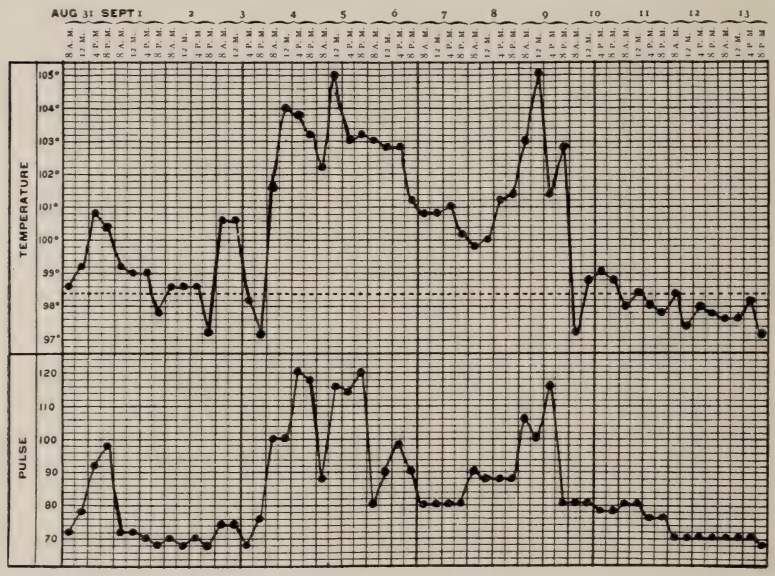


CHART 10.

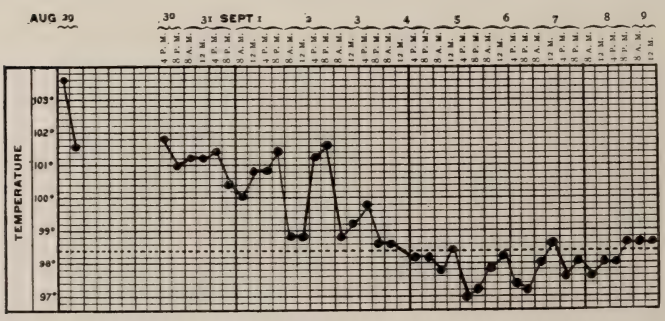


CHART H.

August 31. Inoculated as has been described.
September 1, 2, and 3. Feeling well.
" 4. Had a chill last night about midnight. This morning complains of pain in the muscles and bones, especially of the arms. His eyes ache and are much congested and the face is flushed. Has slight frontal headache and is very nervous. He complains of palpitation of the heart. Tongue moist and clean. Appetite poor.

September 5. Is feeling very nervous this morning, and was delirious last night. Has pain in head, back, arms, and legs. No appetite. Tongue moist, with heavy yellowish coating.

" 6. Spent a restless night, but is not so nervous this morning. Complains of severe pain in the back and legs. There is a faint, slightly elevated, sparse, macular eruption over the chest and back.

" 7. Patient had a comfortable night and this morning has but little pain. The eruption is well marked over the abdomen, chest, back, and arms, and is typical of dengue.

" 8. Feeling very comfortable. The eruption is fading a little.

" 9. Patient was delirious during the early morning hours, and is nervous and restless this morning, but free from pain. The eruption has largely disappeared.

" 10. Began to feel better at 4 P. M. yesterday, and now feels quite well. Slept well but perspired very freely during the night. The eruption has almost disappeared from the body, but is marked upon the forearms and wrists.

" 11. Feeling well. Eruption is fading slowly and there is a slight desquamation in patches.

" 15. Returned to duty.

Remarks.—The symptoms in this case were very severe, especially those connected with the nervous system. The subject of the experiment was of a highly nervous temperament, and this fact accounts, in our opinion, for the severity of the nervous symptoms.

The temperature curve in this case might be used as an illustration of the typical dengue curve, so perfectly does it agree with that described by every observer as characteristic of the disease. It should be noted, however, that the temperature is higher in this case than it usually is in naturally acquired infections or in our other experimental cases, with the exception of Case 9, also produced by the intravenous injection of filtered dengue blood.

We regard these two cases of dengue produced by the intravenous injection of filtered dengue blood as the most typical cases of the severe type of the disease that we have seen, and we believe that these two experiments prove conclusively that dengue can be transmitted by blood which has been passed through a filter which retains organisms as small as 0.05 μ in diameter, the measurement of *M. melitensis*. They also prove, that in all probability, the causative agent is ultramicroscopic in size. It may be possible that in some, as yet, unstudied fluid or organ of the body, or in some phase of its life cycle, the organism may be visible, for Novy, in his work upon *T. lewisi*, has proven that even so large a parasite as is this trypanosome exists in a form so small in cultures that it passes through a Berkefeld filter. While the same may prove to be true as regards the dengue organism, we feel justified in stating that, so far as present evidence goes, the parasite causing dengue fever is ultramicroscopic in size. This conclusion explains the uniformly negative results

obtained by nearly every trained observer in the search for the parasite of dengue. We conclude that a living organism is present in the filtrate, rather than a toxin, because of the length of the period intervening between inoculation and the appearance of symptoms (the incubation period), and also because we have reproduced the disease by the inoculation of the blood of experimental cases.

There is one point deserving of especial consideration in the discussion of these cases of experimental dengue by the injection of filtered blood, and that is, the relatively greater severity of the symptoms present. In both of these cases the symptoms were more intense in almost every particular than in any other of our experimental cases, despite the fact that no greater amount of blood was inoculated in these cases. This fact is very difficult of explanation, and we must confess to our ignorance of the cause. It may be that the admixture with salt solution or the time consumed in filtration, or both, acts in some way to increase the virulence of the organism, or that conditions favorable to its extra-corporeal development are present during the process of filtration which result in a more virulent form of the organism, though we have no evidence to offer in this respect.

Experimental transmission of dengue by the mosquito.—We have already mentioned the experiments of Graham regarding the transmission of dengue by the mosquito, in which he seemed to have proven conclusively that such a method of transmission is possible; we have also noted the negative results obtained by Carpenter and Sutton, Guiteras and Cartaya, and Agramonte, all of whom believe, however, that the mosquito is the active agent in the spread of the disease. To one who studies carefully the epidemiology of dengue, the conclusion is almost inevitable that this disease, which so closely resembles yellow fever and malaria in this respect, must also be transmitted by some species of mosquito. Its seasonal prevalence; its occurrence along low-lying, moist coast regions, and in the valleys of rivers, most frequently; its rapid diffusion in some localities, and its lack of diffusion in others; its relation to changes in temperature and moisture; its manner of spread from building to building in infected places; its absence in high altitudes where mosquitoes are absent; the presence of multitudes of mosquitoes wherever dengue occurs, and the absence of the disease in regions where mosquitoes are absent or few in number,

and the cessation of the epidemic in badly infected districts when conditions arise which are unfavorable to the propagation of mosquitoes, all point to some species of this insect as the infecting agent.

Accordingly, having demonstrated by the intravenous injection of dengue blood that the cause of the disease is present in that fluid, and that the parasite is probably ultramicroscopic in size, we turned our attention to the subject of mosquito transmission. Unfortunately for the fullest success of our work in this direction, we were forced, because of lack of other volunteers, to use a number of men who had already passed unharmed through the epidemic at Fort McKinley, and the majority of whom were immune, as proven by the negative result of inoculation of dengue blood. Thus, of the nine men in whom we endeavored to produce dengue by exposing them to the bites of infected mosquitoes, three were proven in this way to be absolutely immune, one may have had a slight attack of dengue previous to exposure, while three probably possessed a relative immunity, for while they developed dengue from the injection of a comparatively large amount of dengue blood, the symptoms were mild in character, and in one case the incubation period was greatly prolonged. In one case already described (see Case 4), no immunity to the disease existed, but the mosquitoes refused to bite the man.

The mosquito used.—In looking over the geographical distribution of dengue and of various species of mosquitoes, we found but one species of this insect that apparently occurred wherever dengue did, i. e., *Culex fatigans*. We do not wish to be understood as stating conclusively that this mosquito is the only one which may be present in dengue-infected localities, but only that, so far as we have been able to determine from the literature available, this species is constantly found and is mentioned by almost every recent investigator as being very numerous during epidemics of this disease. In Theobald's monograph the map illustrating the distribution of *Culex fatigans* might almost be used to illustrate the distribution of dengue fever, and if to this map be added the regions in which this mosquito has been demonstrated since it was published, the association of dengue and *Culex fatigans* is still more striking

For this reason and because this mosquito was used by Graham in his experiments, we decided to work with this species at first,

and in the event that our results were negative, to extend our work to embrace other species.

We have used mosquitoes reared in captivity, and also those caught in natural surroundings. In our successful case produced by the mosquito, however, we used insects reared by us from the egg, and thus we are sure that no infection occurred in these insects before they bit the dengue patient.

Our mosquito experiments were conducted as follows: The patient suffering from dengue was placed in bed beneath a mosquito-bar in a mosquito-proof tent. At night from 30 to 50 mosquitoes were liberated beneath the mosquito-bar and collected in the morning; almost invariably all of the mosquitoes left alive had bitten and were full of blood. The subject to be experimented upon, having been placed in bed, beneath a mosquito-bar in a mosquito-proof tent, the mosquitoes that had bitten the dengue case the night before were liberated beneath the bar, and orders given that the man remain beneath the bar until the mosquitoes had all disappeared; later we allowed the men to remain out of bed during the day, the mosquitoes being confined beneath the spread bar. With one exception, which has been noted, all of the men were bitten a few times, but in most instances the mosquitoes died or were killed before the men were bitten severely. We also confined mosquitoes that had bitten dengue cases in glass jars, and kept them for from four to six days before allowing them to bite, but in the few instances in which we tried this method our results were all negative.

We do not consider it necessary to give in detail our negative experiments, as they are all referred to elsewhere in this report, and we will only describe here the case in which we were able to produce dengue by the bites of infected mosquitoes.

Experiment 11.

Case 11.—Chart 11. B. L. W. Private, Hospital Corps, U. S. Army. This man had been on duty at the Division Hospital for several weeks, and as no cases of dengue had occurred in the hospital, had not been exposed to the disease. Upon September 12, 1906, the man being in good health, he was placed in bed under a mosquito-bar containing mosquitoes that had bitten Case 88 (Chart R) on the night of September 11, 1906. Case 88 was suffering at the time from a typical attack of dengue and the mosquitoes bit upon the third day of the disease. Case 11 was not bitten by the mosquitoes until the night of September 13, so far as he knows, and developed no symptoms until the night of the 17th, but upon reference to his chart it will be seen that he

had fever for nearly 24 hours before he noticed any symptoms. If we assume the period of incubation to be the period intervening between the 13th, the night upon which he was first bitten, and the 16th, when he had his first rise in temperature, the incubation period would be about $3\frac{1}{2}$ days. If, however, we assume the disease to have commenced when he first noticed symptoms, the incubation period would have been a little over four days. The following is a résumé of the clinical history in this case:

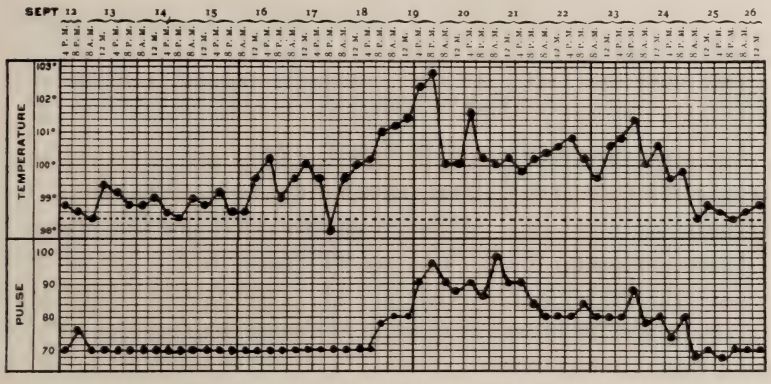


CHART 11.

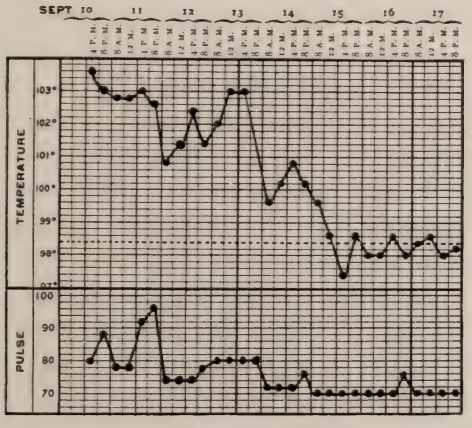


CHART R.

September 12. Put under net with mosquitoes that bit Case 88 last night.
" 14. Bitten by mosquitoes night of the 13th.
" 18. Had headache and felt uncomfortable last evening. This morning complains of headache and dull pain in the muscles and articulations.
" 19. Still complains of headache and general muscular pain and soreness. His face and eyes are greatly congested. Tongue moist and coated with thin white coat in center. Appetite poor. Constipated.

September	20.	Last night had severe pain in the head, eyes, muscles of the back, but feels much better this morning.
"	21.	Is feeling better. A faint rash is visible, covering the chest and abdomen.
"	22.	Complains of soreness and stiffness in the muscles. The eruption is now plainly visible and is a typical dengue eruption.
"	24.	Patient feels well. The eruption has almost disappeared.
October	1.	Returned to duty.

Remarks.—This case was typical, in every way, of a moderately severe attack of dengue. The symptoms were those observed in the great majority of naturally acquired infections, and the temperature chart is a very typical one. This man had not been exposed in our dengue hospital before being bitten by the mosquitoes, and did not leave the mosquito-proof tent until after the onset of the disease.

For reasons which have been stated, of the nine men exposed to the bites of infected mosquitoes, only four can be considered in estimating the results obtained. Of these four, one developed a typical attack of dengue following the bites of infected mosquitoes; but we do not consider that the three negative cases are of much value, as the conditions were such as to cause some doubt as to whether the men were bitten by the mosquitoes.

It is obvious that many factors have to be considered in making mosquito transmission experiments, and it is more than probable that in our negative experiments we were unsuccessful in reproducing the favorable conditions which must have been present in Experiment 11, or the mosquitoes, if they became infected, may have perished before biting again. Schaudinn[9] has called attention to some of the difficulties which may be met with in attempting the experimental transmission of disease by mosquitoes. Thus, certain individuals of a species which has been proven to transmit the disease are not able to transmit it, and this may be due to the insect itself suffering from some other infection; to inability to digest the ingested blood; to an acquired or natural immunity resulting in the death of the specific parasite, or the mosquito may die before it has bitten again.

It is evident from the result of Experiment 11, that the parasite causing dengue does not undergo any cycle of development within the mosquito, unless it be a very short one. We are, therefore, of the belief that the parasite of dengue is one capable of living in the stomach of the mosquito for an unknown period of time, where it retains its virulence; that infection may occur at any time after the insect has ingested blood containing the parasite, so long as it remains virulent,

and that it is introduced into man when the insect bites, being regurgitated through the esophagus and proboscis with the fluid from the stomach. This theory is borne out by the results recently obtained by the Indian Plague Commission[10] in its remarkable study of the transmission of plague from rat to rat by the flea *Pulex cheopis*, and by the excessive rapidity of the diffusion of dengue, which would be impossible were the parasite one which underwent a prolonged developmental cycle within the mosquito.

We have dissected and examined a large number of mosquitoes that had bitten dengue cases, but we have never found any organism in the stomach or tissues suggestive of a stage in the life cycle of a protozoon. We cannot confirm Graham's results in this respect, and we believe that in the mosquito, as well as in the blood of infected man, the dengue parasite is ultramicroscopic in size.

By reason of lack of suitable volunteers and the subsidence of the epidemic, we have been forced to bring our mosquito experiments to a conclusion for the present. We have been unable to investigate many interesting questions regarding the transmission of dengue by the mosquito, such as the length of time the insect remains infective, the most infective period of the disease as regards transmission in this way, and whether transmission is purely mechanical or depends upon the development or multiplication of the parasite within the mosquito. All of these questions are of great importance to a correct conception of the etiology of dengue, and there would appear to be no good reason why, in regions where the disease is common, they should not be thoroughly investigated. We realize that the work that we have been able to do as regards mosquito transmission is very incomplete and that a very great deal remains to be done before this feature of the etiology of dengue is fully elucidated, but we believe that we have confirmed Graham's results in this respect and that we have proven experimentally that this disease can be transmitted by the mosquito, *Culex fatigans*. We also believe that mosquito transmission is the only natural method which has been proven by experiment, and that all of the epidemiological data confirm such a method of transmission.

EXPERIMENTAL PERIOD OF INCUBATION.

As will be seen from a study of the epidemiology of dengue the incubation period has been stated as varying from 24 hours to 10 days,

most observers regarding it to be from three to five days. The following table gives the period of incubation in nine of our experimental cases of the disease:

No. of Case	How Produced	Incubation Period
Case 1	Inoculation of unfiltered blood	3 days 18 hours
" 2	" " " "	2 " 19 "
" 3	" " " "	2 " 18 "
" 4	" " " "	2 " 12 "
" 5	" " " "	4 " 4 "
" 6	" " " "	7 "
" 9	" " filtered "	3 " 11 "
" 10	" " " "	2 " 12 "
" 11	By mosquito. About,	3 " 16 "

From the above table it will be seen that the incubation period of dengue in experimental cases of the disease varied from $2\frac{1}{2}$ days to 7 days, the average being about 3 days and 14 hours. This is practically what is stated by most clinical observers as the incubation period most frequently seen in naturally acquired infections. We have observed no case in which the incubation period was as short as 24 hours, and from our experiments we very much doubt the occurrence of such a short period of incubation.

IMMUNITY AND SUSCEPTIBILITY.

There is considerable confusion existing in regard to these points, the general trend of opinion being that almost everybody is susceptible to dengue, and that an attack produces immunity for a short time only. As to the latter point, i. e., the duration of acquired immunity, we cannot express a very positive opinion, as we endeavored, except in one case, to avoid the use in our experiments of men who had previously had dengue. In the one case noted as an exception dengue was induced though the patient said he had had three attacks, the last, two and one-half years before. We have also known a few other cases in which the disease developed naturally after a like period of time. The correctness of reports of cases in which attacks have been said to occur a month apart we very much doubt. We had about six patients sent back to us after such periods, supposed to be suffering from second attacks, but in no case was it so. The "second attack" was usually a malarial paroxysm.

As to natural immunity, we know that it occurs, or at any rate, that it may be temporarily present. We think it altogether probable that in many cases it may be relative, i. e., a small dose of the virus

may not be sufficient to overcome it, but a large one may. In one of our cases (Case 8) we were unable to decide positively whether an immunity which was present at the time of his discharge was natural or was acquired from a very light attack of the disease following inoculation with a half-minim of blood, though we incline to the latter belief. In at least one instance, immunity was apparent and not real; that is, the patient did not develop dengue when exposed to infected mosquitoes, but it was really due to the fact that the man was immune to mosquito bites. Later he developed dengue from the intravenous injection of dengue blood, and during the time he was sick the mosquitoes bit him as frequently as others about him; fortunately, his immunity to mosquito bites, so valuable in the tropics, was not permanently lost, for the patient now states that he is as free from mosquito bites as before he had dengue.

Our knowledge as to natural immunity cost us rather dear, as we were paying all our subjects of experiment, and did not relish exhausting the funds at our disposal in payments to men not capable of developing the disease. In the light of subsequent events we think that we made a mistake in accepting volunteers from Fort McKinley, where an epidemic of dengue had been and was prevailing, because, while we did not begin experiments upon the men until they had been under our observation and free from exposure for periods varying from a week to three weeks, and thus avoided the error of thinking the disease due to our inoculations when it was in reality due to other causes, we picked men some of whom had probably escaped natural infection because of their natural immunity.

As we have stated, three of our subjects were absolutely immune to dengue. Our assumption that failure to develop the disease after inoculation with 20 minims of blood from a dengue case constitutes absolute immunity is arbitrary, but seems justified by the constancy and severity of the symptoms produced in the successful cases.

Three of the men possibly showed a relative immunity, i. e., the amount of virus transferred to them by mosquitoes was not sufficient to cause the disease, though the intravenous injection of 20 minims of dengue blood was sufficient to do so. Possibly this relative immunity was only apparent, because we know that these men were not severely bitten by mosquitoes, and we do not know that the particular mosqui-

toes that did bite them might not have been laboring under some disability that prevented their transmitting the disease. It is noteworthy that two of these cases suffered from very mild experimental attacks of the disease, and that the third, while an ordinary case, presented an incubation period longer than the average.

Six cases, and if we count the doubtful case already described, seven, presented no immunity; that is, they developed dengue following the first attempt at inoculation. One case, immune to mosquito bites, showed apparent immunity, but developed dengue after the first inoculation.

Natural immunity and the practice of sleeping under mosquito-bars effectually protect a large proportion of healthy men from infection. Thus, in the Fort McKinley epidemic, the highest percentage of infections occurring in any one company was 58 per cent. The next highest was 52 per cent, and in the other companies it was lower. It must be remembered that in this epidemic no special measures were taken to prevent the spread of the disease, and the mosquito protection afforded consisted merely of the ordinary routine use of bars during the sleeping hours.

IMMUNITY AS SHOWN BY EXPERIMENT.

The following cases whose clinical records are here given were proven by experiment to be absolutely immune to dengue. The temperature charts are not reproduced as they contain no data of interest.

Case 12.—W. H. O. First-class private. Hospital Corps. U. S. Army. This man was on duty at the Division Hospital at the time of the experiments, and had never had dengue.

Experiment 1. On the night of September 12 the subject slept under a mosquito-bar with mosquitoes that had bitten a dengue case the night before. No symptoms of dengue developed.

Experiment 2. On the night of September 28 the subject was again exposed to mosquitoes that had bitten a dengue patient the night before. He was bitten repeatedly during the next few nights, but no symptoms of dengue developed.

Experiment 3. On October 3, the subject was inoculated intravenously with 20 minims of unfiltered dengue blood from Case 44. No symptoms of dengue developed, and the man was returned to duty October 11, 1906.

Case 13.—J. G. Private, Co. I, Sixteenth U. S. Infantry. This soldier belonged to a company of the Sixteenth Infantry that had sent twelve cases to hospital with dengue before this man volunteered. He had, therefore, been exposed to the disease.

Experiment 1. On September 12, 1906, the subject rinsed his mouth with normal salt solution containing 12 minims of dengue blood. The result of the experiment was negative.

Experiment 2. On September 19, the subject was given an intravenous inoculation of 20 minims of filtered blood from Case 11 (Chart 11). No symptoms developed

Experiment 3. On the night of October 4, the subject was exposed to mosquitoes that had bitten Case 44 the night before. Dengue did not develop.

Experiment 4. On the night of October 15, the subject was bitten many times by mosquitoes that had bitten a dengue case two nights before. The result was negative.

Experiment 5. On October 22, the subject was given an intravenous injection of 20 minims of unfiltered blood from Case 95 (typical). No symptoms of dengue developed, and the man was returned to duty October 29, 1906.

Case 14.—J. B. P. Private, Co. M., Sixteenth U. S. Infantry. At the time he volunteered the company to which this man belonged had sent ten men to the hospital with dengue.

Experiment 1. On the night of September 24, 1906, the subject was exposed to mosquitoes that had bitten Case 11 (Chart 11) the night before. The result of the experiment was negative.

Experiment 2. The subject was exposed October 26 and 27 to mosquitoes that had bitten a typical case of dengue on October 25. The result of the experiment was negative.

Experiment 3. On November 17 the subject was given an intravenous injection of unfiltered blood from Case 82, who was suffering from a typical attack of dengue. No result attended this experiment and the man was returned to duty November 23, 1906.

Remarks.—These men were all exposed to fomites in addition to the experiments outlined, and we believe that the result of these experiments demonstrates that absolute immunity to dengue is present in certain individuals.

CONTAGION IN DENGUE FEVER.

We have carefully studied this portion of our subject because of its great practical importance, and believe that the following facts conclusively prove that dengue is not contagious in the least degree.

1. At the hospital at Fort William McKinley over six hundred cases of dengue were treated in the general wards without a single case originating among the other patients in the same wards. Only four men belonging to the Hospital Corps on duty at this hospital contracted the disease, three of them being night nurses on duty in the wards and the other a cook having no contact with the dengue patients. No precautions were used to prevent contagion other than the rigid use of mosquito-bars at night, the dengue and other patients eating together, and being closely associated throughout the day. It is noteworthy that the only men unprotected by mosquito-

bars at night, i. e., the three night nurses, all developed the disease.

2. In our dengue hospital, where we treated over 120 cases of the disease, no instance of infection occurred among the attendants, although their association with the dengue patients was very intimate and continued for over four months.

3. Our experiments with fomites were all negative. We endeavored to produce the disease by the exposure of healthy men to fomites, the men experimented with living in mosquito-proof tents with patients suffering from dengue, throughout the entire course of the disease. These men slept in the dengue patients' beds, wore their underclothes and pajamas, and ate and drank from the same table furniture, but in no instance did any of the men develop dengue. In this way we experimented with eight men, in some instances the exposure lasting for as long as two to three weeks.

We conclude, therefore, that dengue is not a contagious disease, and that patients suffering from it may be placed in the general wards of a hospital without fear of spreading the infection, provided precautions are taken to protect the patients from mosquitoes.

CONCLUSIONS REGARDING THE ETIOLOGY OF DENGUE.

From our study of the etiology of dengue, we believe that the following conclusions are justified:

1. No organism, either bacterium or protozoon, can be demonstrated in either fresh or stained specimens of blood with the microscope.

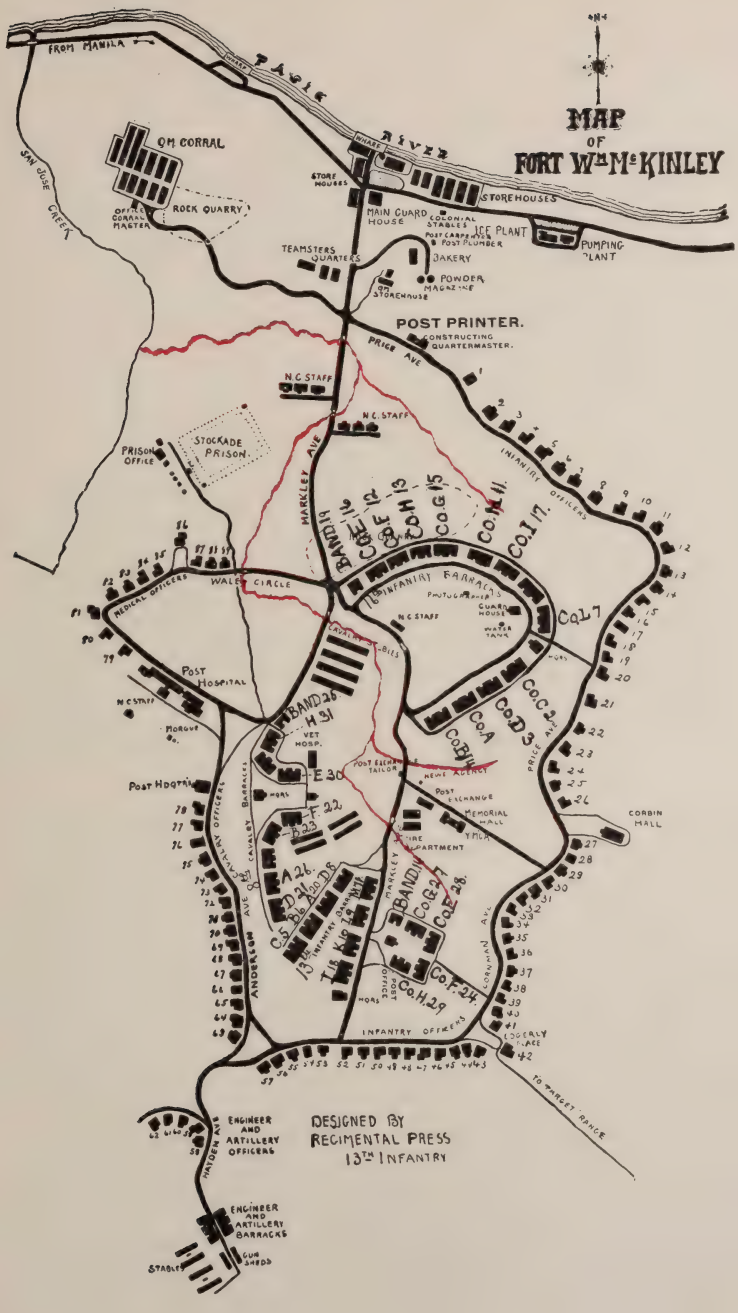
2. The red-blood count in dengue is normal.

3. There occur no characteristic morphological changes in the red or white corpuscles in this disease.

4. Dengue is characterized by a well-marked leucopenia, the polymorphonuclear leucocytes being decreased, as a rule, while there is a marked increase in the small lymphocytes.

5. No organism of etiological significance occurred in broth or citrated blood cultures.

6. The intravenous inoculation of unfiltered dengue blood into healthy men is followed by a typical attack of dengue.


MAP
OF
FORT Wm McKINLEY
FROM MANILA
PACIFIC RIVER
SAN JOSE CREEK
QM CORRAL
ROCK QUARRY
OFFICE CORRAL MASTER
STORE HOUSES
MAIN GUARD HOUSE
COLONIAL STABLES
POST CARPENTER
POST PLUMBER
ICE PLANT
PUMPING PLANT
STOREHOUSES
TEAMSTERS QUARTERS
BAKERY
POWDER MAGAZINE
QM STOREHOUSE
POST PRINTER.
CONSTRUCTING QUARTERMASTER.
PRICE AVE
N.C. STAFF
N.C. STAFF
STOCKADE PRISON.
PRISON OFFICE
MARKLEY AVE
INFANTRY OFFICERS
BAND 19
Co.E 16
Co.F 12
Co.H 13
Co.G 15
Co.M 11
Co.I 17
INFANTRY BARRACKS
PHOTOGRAPHER
GUARD HOUSE
WATER TANK
Co.L 7
N.C. STAFF
MEDICAL OFFICERS
WALE CIRCLE
CAVALRY STABLES
POST HOSPITAL
N.C. STAFF
MORGUE
BAND 26
H 31
VET HOSP.
E 30
POST EXCHANGE TAILOR
NEWS AGENCY
POST EXCHANGE
MEMORIAL HALL
Y.M.C.A.
FIRE DEPARTMENT
Co.C 2
Co.D 3
Co.A
Co.B
PRICE AVE
CORBIN HALL
POST HDQTRS
CAVALRY OFFICERS
CAVALRY BARRACKS
ANDERSON AVE
A 26
D 21
B 26
D 8
C 5
B 6
A 20
D 8
13th INFANTRY BARRACKS
T 18
K 10
L 9
MARKLEY
BAND 19
Co.G 27
Co.E 28
POST OFFICE
Co.F 24
Co.H 29
CORNMAN AVE
INFANTRY OFFICERS
LOGERLY PLACE
TO TARGET RANGE
HAYDEN AVE
ENGINEER AND ARTILLERY OFFICERS
ENGINEER AND ARTILLERY BARRACKS
GUN SHEDS
STABLES
DESIGNED BY
REGIMENTAL PRESS
13th INFANTRY

7. The intravenous inoculation of filtered dengue blood into healthy men is followed by a typical attack of the disease.

8. The cause of the disease is, therefore, probably ultramicroscopic in size.

9. Dengue can be transmitted by the mosquito, *Culex fatigans*, and this is probably the most common method of its transmission.

10. The period of incubation in experimental dengue averages three days and fourteen hours.

11. Certain individuals are absolutely immune to dengue, as proven by our experiments.

12. Dengue is not a contagious disease, but is infectious in the same manner as is yellow fever and the malarial fevers.

In concluding this report we desire to express our appreciation of the encouragement and aid rendered us by Major-General Leonard Wood, commanding the Philippines Division, without which it would have been impossible for us to have made these researches. We also desire to thank Dr. Richard P. Strong, the director of the Biological Department of the Bureau of Science, for the use of apparatus, and Mr. Charles S. Banks, entomologist to the Bureau of Science, who rendered us assistance in the identification of mosquitoes.

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EXPLANATION OF MAP ATTACHED.

The map is intended to illustrate the spread of the epidemic of dengue at Fort William McKinley. The letters placed opposite the barracks indicate the company occupying them, and the figures accompaning the letters indicate the order in which the barracks were infected. The red line indicates the course of a small stream of water which formed an ideal breeding-place for mosquitoes, and which flowed near the barracks.

THE EFFECTS OF EGG-WHITE AND ITS SPLIT PRODUCTS ON ANIMALS; A STUDY OF SUSCEPTIBILITY AND IMMUNITY.*†

VICTOR C. VAUGHAN AND SYBIL MAY WHEELER.

As is shown by the bibliography appended to this paper much of the research work done in the hygienic laboratory of the University of Michigan for some years past has dealt with the detection and extraction of a poisonous group in the proteid molecule. At first we worked with bacterial proteids and later we extended our research to animal and vegetable proteids. In each and all of these we have found poisonous bodies which we have extracted by various methods and obtained in differing degrees of purity. The purpose of this contribution is to report what we have done in the way of splitting up egg-white, to note the effects of the split products on animals, and to correlate the facts observed with those ascertained in our studies of other proteids.

Preparation of the egg-white.—Twenty dozen fresh eggs were broken and the whites dropped into 96 per cent alcohol. The coagulum was frequently stirred and repeatedly extracted with fresh quantities of alcohol. The coagulum was then air-dried on layers of filter paper, pulverized, placed in large Soxhlets, and thoroughly extracted with ether, after which it was again dried, pulverized, and kept in stock in large wide-mouth bottles.

Cleavage of egg-white.—The material prepared after the manner given above is placed in large flasks fitted with reflux condensers and extracted three times with from 15 to 25 times its weight of absolute alcohol in which 2 per cent of sodium hydroxid has been dissolved. These extractions are made at 78° C., the temperature of boiling absolute alcohol. This splits the egg-white into poisonous and non-poisonous portions, the former being soluble in the alcohol, while the latter remains insoluble in this menstruum.

THE POISONOUS PORTION

The alcoholic solution, after separation by filtration from the non-poisonous portion, is carefully neutralized with hydrochloric acid which converts the sodium into a chlorid. The precipitated chlorid having been removed by filtration, the filtrate is evaporated either in vacuo or on the open water-bath. The poison remains as a brownish deposit, containing more or less sodium chlorid, from which it

* Received for publication May 14, 1907.

† Read before the Association of American Physicians, May, 1907.

may be further purified by resolution in absolute alcohol and re-evaporation.

The poison is soluble in absolute alcohol and in water, more readily in the former than in the latter. Its alcoholic solution is perfectly clear, but on standing forms a brownish deposit, which, however, is non-poisonous. An aqueous solution is opalescent, but becomes clear on filtration. The poison, both in powder and in solution, has a peculiar, penetrating, disagreeable odor, which seems to be the same in all the poisons that we have obtained from bacterial, animal, and vegetable proteids. The powder is brownish and deliquescent. Aqueous solutions are decidedly acid to litmus and slowly decompose sodium bicarbonate.

Aqueous solutions of the poison give all the color reactions for proteids except that of Molisch, and the carbohydrate group of the proteid molecule evidently remains in the non-poisonous group in the cleavage process to which we have subjected it. Whether or not the poison is to be regarded as a proteid is a matter that might be discussed. The fact that it is freely soluble in absolute alcohol has a negative bearing which is offset by its response to the color reactions. Its aqueous solutions are precipitated by uranyl acetate and metaphosphoric acid, and when these precipitations are complete the filtrates are not poisonous. The uranyl precipitate is somewhat soluble in alkaline carbonates. We think that the poisonous substance should at present at least be regarded as a proteid. When the poison is heated for some hours with equal parts of alcohol and hydrochloric acid it is destroyed, or, at least, in part robbed of its poisonous properties. From its alcoholic solution the poison is precipitated unchanged by ether.

The poisonous portion of egg-white is purified according to the methods detailed in earlier papers on the subject, and the intensity of its action does not differ, widely at least, from that of the poisons obtained from other proteids. Moreover, the symptoms induced in animals are the same and follow in the same order as when the poisonous portions of other proteids are used. The poisonous groups in all proteids are probably not identical chemically, but consist of closely allied bodies. The poison of egg-white kills just as promptly as that of the colon or typhoid bacillus. There are the same three

stages in the development and progress of the symptoms. At first there are evidences of peripheral irritation, indicated by the unaccustomed and sometimes violent scratching in which the animal indulges. In the second stage there is more or less marked paralysis of the muscles of the trunk and extremities, more noticeable as a rule in the posterior extremities. During this period the animal lies on its abdomen or side and when it moves, it may have some difficulty in co-ordinating its muscular contractions. Finally violent clonic convulsions supervene and death from failure of respiration occurs within from five to sixty minutes after the administration of the poison.

Animals may recover after passing through the first and second stages and after having been apparently very near death; but it is exceedingly rare to have an animal recover after the convulsions have begun. Although the minimum fatal dose is small, ranging from 8 or 10 to 100 mg. given intra-abdominally according to the degree of purification reached, the range between the smallest dose necessary to induce symptoms and that necessary to kill is wide. With one preparation the fatal dose was 70 mg., but 5 mg. developed the first and second stages in well-defined forms. It will be well to bear this in mind for its bearing upon some points that will be developed later in this paper. Like the other proteid poisons, that of egg-white has no apparent effect when administered by the mouth, or when inclosed in collodion sacs, it is introduced into the abdominal cavity.

When the proteid poisons are administered subcutaneously or intra-abdominally and not in large enough doses to cause too speedy death there is a marked fall in the temperature of the animal, but when the dose is overwhelmingly large, or when the poison is administered intravenously, the fall in temperature does not occur. This was noticed by V. C. Vaughan, Jr.,[17] in his study of the action of the intracellular poison of the colon bacillus, and is explained by the suddenness with which the fatal symptoms result. The fall in temperature does not occur, or occurs only rarely, when an animal is killed by a second injection of egg-white, because in this case the poison is liberated within the blood-vessels, and the process is equivalent to an intravenous injection of the free poison. When death is unusually delayed, as it is in some refractory animals sensitized to egg-white, the temperature does fall.

Chronic poisoning with this portion of egg-white or with like portions of other proteids is quite as interesting as the acute form, but as yet we have not given it the attention it deserves. Animals to which repeated non-fatal doses have been given lose flesh, become soft and flabby, are subject to skin eruptions, and die apparently from marasmus. We have seen these effects in guinea-pigs, rabbits, and goats which we have attempted to immunize to these poisons.

THE NON-POISONOUS PORTION.

The part of the egg-white left insoluble in the alcohol is purified after methods given in previous papers, and may be kept either in powder or in solution quite indefinitely without appreciable loss of its characteristic properties. It is largely, not altogether, soluble in water, and its aqueous solutions respond to all the proteid color reactions. The Molisch test, indicating the presence of the carbohydrate group, is given promptly and clearly. So far we have not made any determination of the percentage of carbohydrate in the egg-white residue, but it contains all that originally and naturally exists in the unbroken egg-white. An aqueous solution of this residue is precipitated by strong alcohol, and in this way the residue may be divided into two portions, one soluble and the other insoluble in dilute alcohol. This, as we shall see later, is not a quantitative separation, and each portion is probably a mixture.

INJECTIONS OF EGG-WHITE INTRAPERITONEALLY IN ANIMALS.

A single injection of egg-white into the peritoneal cavity of a rabbit or guinea-pig has no visible effect on the animal. This is true even when the volume is relatively large. Uhlenhuth,* in his studies on specific precipitins, injected at one time into the peritoneal cavity of rabbits the whites of from two to three eggs, diluted to 100 c.c. with physiological salt solution, and found that this treatment was well borne. Indeed, he repeated these injections at intervals of a few days and obtained from the animals thus treated a serum which gave a precipitum when added to egg-albumen diluted with 100,000 volumes of water. This experiment, though in a less heroic way, has been duplicated in many laboratories. We have repeatedly injected into the

* *Deutsch. med. Wchnschr.*, 1900, 26, p. 734.

abdominal cavities of half-grown guinea-pigs 10 c.c. of a dilution of egg-white with an equal volume of either sterilized water or physiological salt solution. We have not had occasion to use larger injections, and it has always seemed that 10 c.c. of fluid is as much as should be injected at one time into the abdominal cavity of a half-grown guinea-pig.

When the peritoneal injections are repeated at intervals of from two to five days for some weeks, the sera of animals thus treated give specific precipitums with solutions of egg-white, and this is regarded as evidence that the animal has acquired the function of digesting and assimilating or otherwise disposing of the foreign proteid introduced in this unusual way into the body. There is no reason for believing that this assumption is incorrect, but recent experiments have given additional interest to this subject.

A paper by Rosenau and Anderson* suggested to us that we might test our poisonous and non-poisonous portions of egg-white obtained as has been stated, by splitting up this substance with dilute alkali dissolved in alcohol.

Rosenau and Anderson found that if an interval of ten days or more elapsed between the first and second injection of serum the effect of the latter upon the animal would be serious and in many instances fatal. These authors say: "The first injection of horse serum has sensitized the animal in such a way as to render it very susceptible to a toxic principle in serum. It is probable that when the guinea-pig is injected with the first, or sensitizing quantity of serum the strange proteid contained in the horse serum develops in the body of the guinea-pig 'antibodies,' which, when brought into contact with more horse serum given at a second injection, produce either a union or a reaction, which cause the toxic action."

Several investigators have noted the peculiar behavior of animals under repeated injections of proteid material of diverse origin. Wolff† injected into rabbits both subcutaneously and intraperitoneally from 3 to 5 c.c. of emulsions of spleen, lymph glands, and bone marrow of calves, and noticed that the first injection was well borne by all, the second by most, but that all the animals died from the effects of the injections, from the third to the fifth, The symptoms observed were practically the same as those we have given as following the injection of our proteid poisons. Otto‡ reported the following findings: (1) With an initial dose of from 0.002 to 0.0025 c.c. of antitoxic serum and diphtheria toxin, an interval of from $4\frac{1}{2}$ to 12 weeks, and a second dose of 6 c.c. of normal horse serum, 50 per cent of the animals died. (2) With a first dose varying from 0.235 to 6.4 c.c. of horse serum with diphtheria toxin, an interval of from six to 14 weeks, and a second dose of 6 c.c. of normal horse serum, all the animals died. (3) With a first injection of horse serum, an interval of from 5 to 10

* *Bull. Hygienic Laboratory*, 29, April, 1906.

† *Centralbl. f. Bakt.*, 1904, 37, p. 390.

‡ *Das Theobald Smithsche Phänomen, v. Leuthold Gedenkschrift.*

weeks, and a second injection of 6 c.c. of rabbit, goat, or ox serum, no reaction occurred in any of the animals. (4) With the first injection of a non-fatal dose of diphtheria toxin, an interval of from four to 11 weeks, of 34 guinea-pigs, 32 gave no reaction and two died. (5) With a first injection of toxone, an interval of from six to 10 weeks, and a second dose of horse serum, no reaction resulted. (6) With a first dose of horse serum varying from 0.0025 to 10 c.c., an interval of from 4½ to 14 weeks, and a second dose of from 6 to 7 c.c. of horse serum, it was found that none of the animals that had a large first dose reacted. Behring and Kitashima* reported the death of a horse during the progress of its immunization, notwithstanding the fact that at the time the serum of the animal was highy antitoxic. There can now be but little, if any, doubt that the horse was sensitized to the proteids contained in the culture medium. Brieger† reported that a goat died of tetanus after having been highly immunized to the toxin of that disease. It would certainly be very easy to mistake the convulsions of an animal dying from a subsequent dose for the spasms of tetanus. Rist‡ says that a guinea-pig bears without apparent injury 0.01 c.gm. of the dried diphtheria bacilli, but 0.05 c.gm. cause progressive loss from which the animal begins to recover in about one week. But if after apparently complete recovery the same dose be given the effect is more marked; paralysis may develop and a third injection of the same amount given a month after the second causes death within from 24 to 48 hours. One pig received intraperitoneally without visible harm 0.01 c.gm. of the dried bacilli; on the 11th day after this it received 0.02 c.gm., which caused loss of weight, but the animal returned to the normal by the 32nd day. On the 36th day the animal received 0.05 c.gm., which led to its death within 24 hours. Arthus§ reported the untoward effects of repeated injections of horse serum in rabbits. He found that a single injection, large or small, caused no visible injury, and the conclusion is that horse serum is not toxic to rabbits. But he also found that a second injection given some days after the first did cause symptoms, light or grave, local or general, immediate or remote, according to the method of giving the second dose, subcutaneously, intra-abdominally, or intravenously, and he concluded that horse serum is toxic to a rabbit sensitized (*anaphylactisé*) to horse serum. The word, "anapylaxie" was first used by Richet and Portier to designate the condition of hypersusceptibility engendered in dogs to the poison of the tentacles of actinia by a previous treatment with the same poison. Later, Arthus and Breton¶ made an interesting report on the skin lesions induced in rabbits by repeated injections of horse serum. Around the point of the subcutaneous injection the tissue became infiltrated and in the more severe cases aseptic gangrene resulted. One of us, 10 years ago, in treating tuberculous individuals with yeast nucelic acid, had more than one fright from the untoward effects of an injection. Immediately after the injection the patient's face would flush and the erythema would rapidly extend over the entire surface of the body. This was accompanied by rapid, shallow breathing. In some instances an urticarial rash followed the erythema and lasted for some hours, but in the majority of instances the untoward symptoms disappeared almost as rapidly as they came and within a few minutes the patient was able to arise and walk home. At that time it was supposed that the effects were due to unintentional

**Berl. lin. Wchnschr.*, 1901, 38, p. 157.

† Quoted by Otto, p. 9.

‡ *Comp. Rend. de la soc. biol..*, 1903, 55, p. 978.

§ *Comp. Rend. de la soc. biol.*, 1903, 55, p. 817.

¶ *Comp. rend. de la soc. biol.*, 1903, 55, p. 1478.

intravenous injections, but the fact is now recalled that these symptoms were most frequently observed when several days elapsed between injections.

As we are writing this, Dr. Cumming, the assistant in this laboratory in charge of the Pasteur treatment of rabies, calls our attention to the following interesting case: E. M., of Dayton, O., aged 13, was treated in January, 1906, on account of the bite of a rabid dog. In March, 1907, the boy returned, having been bitten by a rabid cat. No unusual symptoms developed in the first treatment, but in the second treatment nothing was observed until the fourth day, when there developed about the point of injection an area of aseptic inflammation about three inches in diameter. The swelling became noticeable about six to seven hours after the injection and gradually disappeared after 48 hours. This resulted from every injection subsequent to the fourth. The treatment was continued notwithstanding these local effects and no harm seemed to be done. The sister of this boy had her first treatment at the same time that he was having the second, and received the same emulsions and in the same amount without any local or other reaction.

The effects of repeated injections of antidiphtherial serum, with intervals of from several days to years between treatments, have been reported tby Otto (*loc. cit.*), by Pirquet and Shick,* Rolleston,† and Currie.‡ The most elaborate and valuable of these reports are those of Pirquet and Shick. It should be stated that no fatal result has been reported due to repeated injections of antidiphtherial serum, but grave symptoms have been observed. Pirquet and Shick reported 61 cases with the interval between treatments of from 12 days to 7½ years. Under 12 days there is no reaction. In 30 cases in which the interval was between 12 and 50 days all showed an immediate reaction. In 11 cases with an interval of from two to six months there were both immediate and accelerated reactions and in 19 cases with an interval of from seven months to 7½ years there was an accelerated reaction. By an accelerated reaction is meant the development of symptoms which may follow a single injection, but which come on more promptly and are more severe.

Besredka and Steinhardt§ have made an interesting contribution to the literature of hypersusceptibility. They believe that the first injection of horse serum, although apparently harmless, produces a brain lesion, which renders this organ especially susceptible to the second injection, and they prove this, apparently to their own satisfaction, by showing that the second dose is more surely fatal when given subdurally, but they fail to explain why a period of from 10 to 12 days must elapse before this brain lesion becomes susceptible to the second dose. Furthermore, they have no difficulty in establishing what they call immunity or a condition of antisusceptibility. One of their experiments is detailed as follows: Two guinea-pigs received eight days after sensitization an intraperitoneal injection of 5 c.c. of horse serum without being affected. One received 10 days later and the other 14 days later subdurally 0.25 c.c. of serum and, as a result of this both animals, although visibly incommoded, soon recovered, while a control that had not received the second intraperitoneal injection—which they call a vaccination—died within five minutes. They might have observed the same phenomenon had the third dose been given intra-abdominally or subcutaneously. However, the contribution is a valuable one and shows two things: (1) the interval

* "Die Serum-krankheit 1905," *Münch. med. Wchnschr.*, 1906, 53, p. 67.

† *Practitioner*, 1905, 74, p. 664.

‡ *Jour. Hyg.*, 1907, 7, p. 35.

§ *Ann. de l'Inst. Past.*, 1907, 21, p. 117.

between the first and second dose must, in order to establish full sensitization, be as long when the second dose is given subdurally as when it is given subcutaneously or intra-abdominally. (2) Their results indicate that the poison that kills a sensitized animal does so by its action on the brain. We have repeatedly emphasized this second point in previous papers on the action of the poison obtained in a free state by splitting up the proteid molecule in the retort. Nicolle* has made a valuable report, but as it deals with repeated injections with short intervals it has but little bearing on the points that we are to discuss and we refer the reader to the original.

In our experiments with egg-white we have used material obtained directly from fresh eggs and the split products secured by the method already given. The fresh material has been drawn from newly laid eggs, diluted with an equal volume of either sterilized water or salt solution and filtered through paper. Our most important results may be formulated as follows:

1. One injection of egg-white is without apparent effect upon guinea-pigs, but sensitizes these animals after an interval of 10 to 12 days to a second injection. This is shown by the following:

TABLE 1.

All these animals received a sensitizing dose of 5 c.c. of the egg-white dilution and a second dose of the same amount, leaving the time interval between the injections the only controllable variable.

No.	Gms. Weight	No. Days Interval	Result
360	315	2	Not affected
361	335	4	" "
363	325	6	" "
368	320	8	" "
1	275	12	Died within 40 min.
5	355	27	" " 40 "
33	495	62	" " 30 "
28	305	92	" " 20 "
56	485	125	" " 20 "
52	310	154	" " 18 "

2. One c.c. of the egg-white dilution serves as a sensitizing dose, just as well as five times that amount.

It will be seen by comparing Tables 1 and 2 that, notwithstanding the difference in the sensitizing doses, the effects are the same. There is the same interval between the time of administration of the sensitizing dose and the development in the animal of the condition of sensitization, and when developed this seems to be the same both in intensity and in permanency.

3. However, with wider ranges in the size of the sensitizing doses differences in effect may be observed.

* *Ibid.*, p. 128.

TABLE 2.

These animals received a sensitizing dose of 1 c.c. and a second dose of 5 c.c. of the egg-white dilution (1:1).

No.	Gms. Weight	No. Days Interval	Result
85	305	6	Not affected
86	225	8	" "
87	235	10	1st and 2d stages
88	235	12	Dead in 15 minutes
4	355	19	" " 15 "
8	275	26	" " 15 "
89	385	37	" " 65 "
91	290	47	" " 15 "
92	285	63	" " 50 "
93	230	79	" " 40 "
94	280	93	" " 8 "
95	280	105	" " 6 "
96	295	124	" " 12 "
97	245	152	" " 20 "

In studying the sensitization of animals the following factors should be taken into consideration: (1) The amount of the sensitizing dose. (2) The interval in time between the first and second doses. (3) The amount of the second dose. (4) The individuality of the animal. In the following table we have arranged a series according to the size of the sensitizing dose. The animals were all of practically like age at the time of sensitization. The second dose was the same in all. The two controllable variables are the size of the sensitizing dose and the time interval. It should be stated that the weights given in this and in all our lists are those of the animals at the time they received the sensitizing doses, and the amount of egg-white given is that of a dilution with an equal volume of either sterile salt solution or water, filtered through paper.

TABLE 3.

No.	Gms. Weight	1st Dose in c.c.	No. Days Interval	2d Dose in in c.c.	Result
52	310	0.02	13	5	1st and 2d stages
51	285	0.05	13	5	1st " 2d "
50	240	0.01	13	5	1st " 2d "
49	285	0.20	13	5	1st " 2d "
88	235	1	12	5	Dead in 15 min.
4	355	1	19	5	" " 15 "
7	285	2	13	5	" " 30 "
16	300	2	33	5	" " 15 "
3	315	2	13	5	1st and 2d stages
2	305	3	13	5	1st " 2d "
6	305	3	13	5	Dead in 30 min.
11	260	4	31	5	" " 40 "
15	290	4	39	5	" " 15 "
1	275	5	12	5	" " 40 "
28	305	5	92	5	" " 20 "
10	350	6	33	5	" " 35 "
14	360	6	45	5	" " 17 "
9	370	10	18	5	1st and 2d stages
13	335	10	56	5	Dead in 20 min.
21	305	10	13	5	1st and 2d stages

We are well aware of the fact that before positive and final conclusions are drawn the list must be a much longer one, but the following conclusions may be formulated tentatively: (1) The very small sensitizing doses, under 1 c.c. of the dilution, are not so efficient as the larger ones. (2) One c.c. of the dilution is quite as efficient as a sensitizing dose as a larger one. (3) The time interval is an important factor. (4) When the largest sensitizing doses are used the time interval must be longer in order for the second dose to kill.

4. The non-poisonous portion of egg-white, designated as "the residue," sensitizes to unbroken egg-white.

TABLE 4.

These animals received varying amounts of the residue as sensitizing doses, but all received 5 c.c. of the egg-white dilution (1 : 1) for the second dose. They are arranged according to the time interval.

No.	Gms. Weight	Amt. o. Res. in mg.	No. Days Interval	Result
99	295	25	4	Not affected
100	245	25	4	" "
101	240	25	6	" "
56	220	10	10	1st and 2d stages
55	260	25	10	Died in 12 min.
102	340	25	10	" " 18 "
54	295	50	10	Not affected
120	395	50	10	" "
53	285	100	10	Died in 15 min.
61*	260	50	11	Convulsions, but recovered
62	305	50	13	Died in 36 min.
103	290	25	15	" " 40 "
106	355	25	15	" " 15 "
121	420	50	15	" " 25 "
20	325	200	15	" " 20 "
19	340	300	15	" " 15 "
63	335	50	25	" " 17 "
64	315	50	25	" " 40 "
183	485	1	26	Not affected
185	570	1	26	Slightly affected
186	575	1	26	1st and 2d stages
184	605	1	26	Died in 23 min.
180	615	5	26	Not affected
179	495	5	26	Slightly affected
182	425	5	26	Died in 27 min.
175	515	10	26	" " 20 "
123	465	50	31	" " 15 "
124	655	50	41	Not affected
125	355	50	41	Died in 15 min.
126	660	50	41	Not affected
104	305	25	114	Died in 18 min.
105	315	25	114	" " 30 "
119	370	50	114	" " 15 "
132	390	50	114	" " 20 "

It will be noticed, in looking over this table, that there are a few animals, which even after an interval of 10 days or more are not sensitized. This occurs when unbroken egg-white is used for the sensitizing dose quite as frequently as it does when the non-poisonous

* No. 61 is the only animal that we have seen recover after convulsions appeared.

portion is employed for this purpose. We are of the opinion that the failure to sensitize with either the non-poisonous portion or the unbroken egg-white is due to the age of the animal, and that old animals are not so easily sensitized as the young. It will be observed on going over the table that all the animals that were not affected by the second dose after the proper time interval were old animals, as indicated by their weight. It is true that some old animals were sensitized, but it is equally true that all those that were not sensitized were old. We state this only as an opinion, and are fully aware of the fact that a much greater number of animals would be required to make a positive demonstration. If this opinion should prove to be a fact it will probably be found that the small sensitizing doses will prove much more efficient when young animals are used. It happened quite accidentally that the animals in which we used the smallest sensitizing doses were some which we secured because they were believed to be too old to breed and all were old males. At the time that we sensitized these animals we had not thought of age as a factor in sensitization, and it only occurred to us that this might be true after we had tabulated the results. This point is an interesting one and deserves further study.

5. The non-poisonous portion of the residue does not sensitize to itself as is shown by the following:

TABLE 5.

No.	Gms. Weight	1st Dose in mg.	No. Days Interval	2d Dose in mg.	Result
103.....	290	25	10	150	Not affected
104.....	305	25	15	250	" "
105.....	315	25	15	200	" "
119.....	370	50	10	250	" "
122.....	390	50	15	250	" "

No. 103 received five days after the second injection of the residue 5 c.c. of the egg-white dilution, from which it developed the characteristic symptoms and died in 40 minutes. This shows that the animal was sensitized by the first dose of the residue, because the sensitized condition requires more than five days for its development. Moreover, another animal (106) received 25 mg. of the residue at the same time, and 15 days later it had 5 c.c. of egg-white dilution from which

it died in 15 minutes. Nos. 104, 105, 119, and 122 received, 114 days after the sensitizing dose of the residue was given, 5 c.c. of egg-white dilution and died in 18, 30, 15, and 20 minutes, respectively. No. 103 suggests another interesting point, i. e., the second dose of the residue did not prevent the animal from splitting up unbroken egg-white given five days later, which would have been the effect had the second dose been a non-fatal one of egg-white.

The fact that the residue does not even in the slightest degree sensitize to itself, while it does fully sensitize to unbroken egg-white, demonstrates two things very clearly. First, it shows that our separation of the poisonous and non-poisonous portions is an actual and complete separation. If there remained in our non-poisonous portion any unbroken egg-white it should sensitize to itself, and the second dose, especially with the large quantities that we have used, should develop some symptoms even if it does not kill, for we have seen that one-fourteenth of the fatal dose of the poison develops to a marked degree the first and second stages. In the second place, it shows that only when the second dose contains the poisonous group does it develop symptoms. This, along with the fact that the symptoms induced by the split-off poison and those that follow the injection of unbroken egg-white into a sensitized animal are identical, proves conclusively, to us at least, that sensitization consists in rendering the animal capable of splitting up egg-white and that this cleavage as it occurs in the animal yields the same products that we obtain by our artificial method in the retort. Our method is undoubtedly crude compared with the cleavage process that occurs in the body of the sensitized animal, so far as quantity of active poison is concerned, for we are quite sure that by the artificial method we destroy more or less of the poison; but, so far as the quality of the poison yielded by the two processes goes, the results are identical.

6. There are reasons for suspecting that the sensitizer contains two bodies the joint presence of which is necessary for full sensitization.

We speak with caution on this point and recognize that additional work is necessary before we can make a positive statement. An aqueous solution of the residue was acidified with hydrochloric acid and treated with an equal volume of absolute alcohol. The pre-

cipitate produced by this treatment was collected and dried and the filtrate evaporated to dryness and these portions were employed separately and jointly in sensitizing animals with the following results.

TABLE 6.

No.	Gms. Weight	Amount in mg.	No. days Interval	No. c.c. of Egg-White	Result
			PRECIPITATE.		
262	305		11	5	Not affected
263	370		25	5	1st and 2d stages
264	405		11	5	Slightly affected
			FILTRATE.		
265	415	100	10	5	Slightly affected
266	405	50	24	5	1st and 2d stages
267	425	25	24	5	1st " 2d "
268	455	12.5	10	5	Slightly affected
			COMBINATION.		
318	350	25 each	12	5	Died in 5 min.
319	360	25 "	12	5	" " 45 "

We have obtained similar results in immunizing animals to the colon bacillus with the non-poisonous part of the cellular substance of that bacterium; but, as we have stated, this point deserves further investigation.

7. The active substance in the sensitizer or residue is probably a proteid.

The evidence upon which this statement is founded was obtained from experiments in which all proteid was removed from aqueous solutions of the residue after which it failed to sensitize. After complete precipitation of an aqueous solution of the residue with uranyl acetate and removal of uranium from the filtrate with sodium phosphate, the portion of the residue left failed to sensitize animals. We have inserted the word "probably" in our statement, because we recognize that there remains the possibility that the sensitization may be due to some non-proteid group which is precipitated with the proteid by uranyl acetate. But we have found that whenever we remove from our residue the group or groups that give the proteid color reactions, it fails to sensitize.

8. The poisonous portion of the split products of egg-white either in single or in multiple doses, does not sensitize to unbroken egg-white.

TABLE 7.

These animals had 5 c.c. of the egg-white dilution after previous treatment with the poison.

No.	Gms. Weight	Amt. Poison in mg.	No. Days Interval	Result
41	490	12.5	36	Not affected
45	265	5	37	" "
33	495	25	11	" "

Other animals had multiple injections of the poison and after varying intervals from the time of the last injection received 5 c.c. of the egg-white dilution without effect. This demonstrates that no portion of the sensitizing group remains in the poisonous portion obtained by our method of separation. This test is more delicate than a chemical one, for had one mg. of the residue remained in the poisonous portion it would have sensitized the animals to unbroken egg-white, and some of our pigs that received multiple injections of poison were given altogether as much as 275 mg. of the poisonous portion. We, therefore, conclude that these experiments demonstrate two things: (1) The poisonous portion does not sensitize to unbroken egg-white, and (2) the poisonous portion as we have used it contains no trace of the sensitizing substance.

9. The second dose in order to kill must contain enough egg-white to furnish a fatal dose when split up in the animal body.

Inasmuch as the cleavage process in the animal body occurs in part at least in the blood, the amount of poison necessary to kill under these conditions is small—probably less than 5 mg., possibly less than half this amount—because it has been shown in our work on the colon poison that 10 mg. of the crude poison may kill within four min., when given intravenously, and we have learned that the pure poison does not constitute more than 15 per cent of the crude product. This would reduce the fatal dose when given intravenously to 1.5 mg. The minimum fatal dose cannot be greater than this and it may be smaller. It follows from this that the second dose may be small and yet cause a fatal result, but that it cannot be infinitesimally small and cause death. Indeed, the second dose of egg-white, in order to kill, must be an easily measurable quantity. There is no reason, so far as we can see, for holding or supposing that the second dose must act like a ferment, because it may be very small and yet kill. Whether or not the poison kills depends not only on the amount injected into

the animal body but the rapidity with which it is introduced. We have seen that this is so important a factor that when a quantity of the poison equivalent to several times a fatal dose measured by the effects when injected intravenously, intra-abdominally, or subcutaneously, is introduced into the stomach or sacked in the peritoneal cavity, it has no visible effect on the animal although the poison slowly diffuses unchanged through a collodion sac. Although we regard the effect of an intra-abdominal injection of egg-white into a sensitized guinea-pig as comparable in some respects to an intravenous injection of the free poison, we recognize that there are differences, and we would not expect an amount of egg-white which would furnish not more than 1.5 mg. of the free poison to kill a sensitized animal. In other words, if the minimum fatal dose of the poison when given intravenously be 1.5 mg., we would expect that the minimum fatal dose of egg-white in a sensitized animal would be an amount which on being split up would yield something more than this amount of the poison.

TABLE 8.

No.	Gms. Weight	1st Dose in c.c.	No. Days Interval	2d Dose in c.c.	Result
179	495	5	17	1	Slightly affected
180	615	5	17	1	" "
183	485	5	17	1	1st and 2d stages
185	570	5	17	1	1st " 2d "
380	470	5	18	1	1st " 2d "
381	485	5	18	1	1st " 2d "
382	475	5	18	1	1st " 2d "
383	465	5	18	1	1st " 2d "
384	445	5	18	1	1st " 2d "
385	440	5	18	1	1st " 2d "
386	500	5	18	1	1st " 2d "
387	490	5	18	1	1st " 2d "
388	515	5	18	1	1st " 2d "
389	515	5	18	1	1st " 2d "

Although egg-white when injected intra-abdominally in a sensitized animal acts speedily, its average action cannot be so rapid as that of the free poison injected directly into the blood. We have found, as the next table shows, that 1 c.c. of the egg-white dilution (1:1) does not kill adult sensitized pigs. Now 1 c.c. of this dilution contains not more than 500 mg. of egg-white and egg-white contains about 12 per cent of proteid. This egg-white does not contain so much proteid because it has been filtered through paper and a considerable proportion of the proteid has not passed through the paper.

But supposing that it did contain 12 per cent of proteid, then our injection contained 60 mg. of proteid, and we have shown that egg-white yields about one-third its weight of crude poison, and this would make 20 mg., and 15 per cent of this would be 3 mg., which must represent the maximum of pure poison that the 1 c.c. of the dilution could contain—it actually did not contain so much. It should be remarked that all proteids do not yield like amounts of the poisonous groups.

In comparing this list with others it must be evident that the size of the second dose has an important influence on the result, and that 1 c.c. of our egg-white dilution when split up in the body of an adult pig does not supply a fatal dose of the poison.

10. Animals that are sensitized and yet recover from the second injection of egg-white are affected by and may die from a third or a subsequent injection, provided the interval between treatments is long enough.

The animals in the following table illustrate this statement. The sensitizing doses ran from 2 to 10 c.c. of the egg-white dilution but all the subsequent doses were of the same size, except the last one given No. 3. It appears from this table that there is a minimum interval which must supervene between any two injections before the animal regains the sensitized state. Whether or not this interval is longer than that which is necessary between the first and second doses we have not sufficient experimental data for determining, but the indications are that the interval must increase with the number of treatments. With No. 2, the interval between the second and third doses was six days, and this was not enough for the animal to regain its condition of sensitization; but in all other instances in this list the animal was in a sensitized state when the dose was given it. A pig need not die in order to show that it is sensitized; the development of the first and second stages is sufficiently characteristic to demonstrate sensitization. The question why the ordinarily fatal dose does not kill the sensitized animal is apart from that whether or not the animal is sensitized. The sixth dose of egg-white made No. 2 quite as sick as the second one, and on the receipt of each the animal was in a sensitized state; indeed, the animal finally died from the sixth dose. Now, why was it that 5 c.c. of the egg-white dilution

did not kill any of these animals the first time it was given them after they had been sensitized? If this question can be answered satisfactorily it will also tell us why it failed to kill at any of the subsequent injections. The most ready answer to the question is that the 5 c.c. of the egg-white dilution failed to kill these pigs because it did not contain enough poison. The dose contained the amount of poison that usually kills sensitized pigs, but these are unusually resistant and do not die. Increase the dose of the egg-white and the animals will die. We did increase the dose in No. 3, and yet the animal did not die. The second dose must furnish a fatal amount of the poison when the proteid is broken up in order to kill, but the proteid may contain several times the fatal dose and still not kill the sensitized animal. In our opinion this is due to the rate at which the proteid is broken up and the poison set free. Animals that prove refractory after sensitization are those in which the proteolytic process for some reason proceeds slowly. We have observed that these refractory animals develop even the first stage of poisoning much later than it usually appears, and the second stage lingers a long time and these animals may die without passing through the convulsive stage. We have in these animals what we may designate as a subacute form of poisoning, and they die no quicker from 10 c.c. of the egg-white dilution than they do from half that amount. Indeed, we are not sure that the failure to kill No. 3 was not due to the fact that we doubled the last dose, but this brings up a question which we are not at present prepared to discuss and concerning which we hope to be able to speak more confidently soon.

11. The young born to sensitized parents inherit the condition of sensitization. We reserve the discussion of this point for a future paper.

Attempts were made to ascertain whether or not the ferment supposed to break up this egg-white could be detected in the body of a pig just dead from the effects of a second dose. These experiments while not wholly satisfactory are worthy of record and deserve repetition and extension.

The sterile salt washing from the peritoneal cavity of a pig just dead from a second dose was added to 10 c.c. of the egg white dilution, and the mixture after standing for 16 hours at 37° was injected

into the abdominal cavity of a fresh pig. The animal developed the first and second stages of poisoning but recovered.

TABLE 9.

No.	Gms. Weight	1st Dose in c.c.	No. Days Interval	Subsequent Dose in c.c.	Result
2.....	305	3	13	5	1st and 2d stages
			6	5	No effect
			21	5	1st and 2d stages
			30	5	1st " 2d "
			96	5	Died in 2½ hours*
3.....	315	2	13	5	1st and 2d stages
			16	5	1st " 2d "
			41	5	1st " 2d "
			96	10	1st " 2d "
5.....	355	5	13	5	1st " 2d "
			27	5	Died in 40 min.
9.....	370	10	18	5	1st and 2d stages
			27	5	Died in 45 min.
21.....	305	10	13	5	1st and 2d stages
			44	5	1st " 2d "
			96	5	Died in 56 min.
23.....	310	5	13	5	1st and 2d stages
			44	5	Died in 30 min.
24.....	295	5	13	5	1st and 2d stages
			44	5	1st " 2d "
			96	5	Died in 35 min.
25.....	310	5	13	5	1st and 2d stages
			44	5	Died in 20 min.

* No. 2 died more slowly than any other animal that we have killed with egg-white.

Five c.c. of the egg-white dilution mixed with the same amount of peritoneal washing from a pig just dead from a second dose was immediately injected into the abdominal cavity of a fresh pig. This animal developed the first and second stages but recovered.

Five c.c. of the egg-white dilution was rubbed up with the spleen of a pig just dead from a second dose and the fluid portion was immediately injected into the abdominal cavity of a fresh pig: No effect. Like results followed similar treatments with the adrenals and portions of the omentum and liver. We washed out the peritoneal cavities of three pigs just dead from second doses of egg-white with sterile salt solution and immediately injected these washings into the abdominal cavities of three fresh pigs. These animals immediately became restless, scratched, and kept up a continuous crying for some minutes and speedily recovered. The object in this experiment was to demonstrate, if possible, the existence of the free poison in the abdominal cavity of the dead guinea-pig, but the evidence was not positive and still leaves us in doubt. However, we could not expect to find any more than a trace of the poison in the peritoneal cavity of an animal dead from its effects. It is worthy of note that the ani-

mals treated with these peritoneal washings were thereby sensitized to unbroken egg-white, as was demonstrated by subsequent treatment. The sensitization may have been due to either whole egg-white or to the non-poisonous portion.

CHRONIC POISONING WITH EGG-WHITE.

It is not our purpose to enter minutely in this paper into the interesting subject of chronic poisoning with foreign proteids, but some general statements should be made. We have treated guinea-pigs daily with intra-abdominally injections of egg-white dilution (1:1). One set of pigs received daily 5 c.c. and another 2 c.c. of the dilution. These animals were apparently but little affected. Most of them lost weight, but some did not and others fluctuated, losing for a few days and then gaining. Two died after the eighteenth, six after the nineteenth and one after the twenty-first injection. It should be stated that those having 2 c.c. of the egg-white dilution died quite as promptly as those having 5 c.c. The post-mortem finding was uniform in all and quite interesting. It consisted of a hemorrhagic, aseptic inflammation of the omentum. Besides the omentum and the mesentery all other organs, macroscopically at least, appeared normal. There was in all instances a bloody fluid in the peritoneal cavity, and this was sometimes cloudy with coagulated fibrin. The parietal peritoneum was normal. It should be stated that paralysis and convulsions precede death much as they do after acute poisoning, but continue through a much longer time. If the body be properly sectioned, cultures made from the peritoneal cavity, omentum, liver, spleen, and heart's blood remain sterile. The localization of the inflammation in the omentum and mesentery is striking and characteristic. Evidently the continued absorption of the foreign proteids lead to congestion and finally to hemorrhage.

An animal that has received twenty of the smaller doses gets altogether only $2\frac{1}{2}$ grams of albumen, and this given in divided doses kills. It must be evident from this that the repeated injection of foreign proteids into animals cannot be regarded as altogether free from ill effect.

INTERPRETATION OF RESULTS.

A correct interpretation of the phenomena which have been recorded in the preceding pages will be of much value to both the physiologist

and the pathologist; and at the risk of falling into error this will be attempted, with the provision that further study and elucidation of the facts, may lead to a modification of their explanation. We have by chemical agents outside of the animal body broken up the complex proteid molecule into two groups, one of which contains a poisonous substance, while the other group contains a body which sensitizes animals to the unbroken proteid. We, probably better than others, are fully conscious of the fact that our artificial method of splitting up the proteid is crude compared with the process that takes place in the body of the sensitized animal. We called attention to this some years ago, in discussing this question in connection with our work on the cellular substance of the colon bacillus and its cleavage products. At that time we stated that by our artificial method much of the poisonous body must be destroyed, or at least rendered inert, while in the animal body this does not occur, at least not to so great an extent. We do not suppose that by our artificial method we have broken up the proteid molecule into two and only two parts, one of which is haptophor or possessed of sensitizing properties, and the other toxophor, or a chemically pure body possessed of poisonous properties. Indeed, we know that this is not true, and that one group contains other than the haptophor and that the other contains, in addition to the toxophor, substances that are inert. Our work shows conclusively that our non-poisonous residue contains besides the sensitizing bodies substances that have nothing to do with inducing in the animal the condition which for the want of a better term we call sensitization, and with equal certainty our work shows that our poisonous substance is a mixture and not a chemically pure body. As yet we remain quite ignorant of the chemical composition and constitution of both the sensitizer and the poison, and the solution of these questions is a task still before us. However, with what has been done chemically outside of the body and with what has been learned by animal experimentation, we think that we have some basis upon which a theory may be, at least tentatively, offered. Furthermore, the observation of facts, the prosecution of laboratory investigations, and the sacrifice of animal life in experimentation are of little value unless we attempt to correlate the facts, systematize the chemical investigations, and study the relationship between cause and effect in our animal experimentation.

We can see no escape from the conclusion that the active agent in our toxophor obtained by chemical means from egg-white, and the substance that kills the animal sensitized to egg-white, when a second dose of this is administered, is one and the same. Both are constituents of the egg-white, and are groups in the same complex molecule. However, if one wishes to contend that egg-white is not made up of complex molecules, as we hold, but is a physical mixture of substances among which our haptophor and toxophor exist, he still cannot avoid the conclusion stated in the preceding sentence any more than he can escape the conviction that the man who dies from morphin and the one who dies from opium die from the same poison. The symptoms induced by the toxophor split off by chemical agents and those observed in the animal sensitized to egg-white on the second administration of this substance are identical in every particular. They originate in the same time, proceed in like order, and terminate alike. The mode of death is the same and the post-mortem findings in both are identical. We must, therefore, conclude that the process of sensitizing an animal consists in developing in its body a substance which affects the egg-white, just as the alcoholic solution of alkali does in the retort, but much more promptly and efficiently.

It is equally certain that our artificially obtained haptophor contains the substance which develops in the body of the animal the capability of speedily and effectively splitting up the egg-white, or, if one prefers, extracting and liberating its poisonous constituent. This is shown by the fact that our haptophor sensitizes animals to the poisonous action of egg-white quite as well as egg-white itself does. The poison in egg-white therefore has nothing to do with the sensitization of the animal, and that there is no poison left in our artificially prepared haptophor is shown by the demonstration that it does not sensitize to itself. It seems that the demonstration of these points is complete and incontrovertible.

That the egg-white is split up in the body of the sensitized animal is shown by the experiments in which unsensitized animals were affected by the injection of the peritoneal washings from sensitized animals just dead from injections of egg-white. We will admit that the evidence on this point has not been so clear and striking as we might wish, but it could not be expected that large amounts of the

free poison would be found in the peritoneal cavity of an animal just dead from its effects, any more than we should hope to recover from the stomach of a man who had just died from the minimum fatal dose of morphin an equal amount of that poison. That either unbroken egg-white or its haptophor remains in the peritoneal cavity of an animal dead from the effects of egg-white is shown by the fact, repeatedly demonstrated, that the washing from this cavity sensitizes fresh animals to egg-white.

That the cleavage agent does not exist, at least in effective amount, in the liver, spleen, adrenals, or omentum of the sensitized animals is indicated by the results of experiments already detailed. That the cleavage agent does not exist, during the intervals between injections, in the blood of the sensitized animal we infer from the failure to detect any trace of it in the blood serum obtained from sensitized animals during these intervals.

There is no evidence that an antitoxin is produced by single or repeated injections of either the unbroken egg-white, its toxophor, or its haptophor constituents. It is true that some slight increase in the resistance of the animal to the toxophor may be induced by repeated injections of this body seems well established, but that this is due to the production of an antibody we see no good reasons for believing. In the first place, this increased tolerance is at best only slight, it is not specific, and the attempt to detect an antibody in the blood serum of sensitized animals has uniformly failed. A sensitized animal is no more and no less susceptible to the toxophor than a fresh animal. Indeed, we can see absolutely no ground for believing that the toxophor has any concern in inducing the condition of susceptibility which we call sensitization. Quite naturally it occurred to us that the haptophor might elaborate an antibody to its own toxophor, but by neither single nor repeated injections of the haptophor or the unbroken egg-white have we been able to secure any confirmation of this possibility.

It seems that we must conclude that the introduction of egg-white or its haptophor constituent into the blood of an animal, by either intra-abdominal or subcutaneous injection leads to the production of a new digestive or proteolytic secretion. It leads to the development of a new function on the part of certain body cells, and

this new function consists in the elaboration of a secretion which breaks up egg-white in the animal body, very much as we have broken it up with the alcoholic solution of alkali but much more quickly and efficiently. This new proteolytic secretion is formed and held in certain cells in the body until these are stimulated by the reappearance of egg-white in the circulatory blood. This secretion belongs to that class of bodies which have long been designated as ferments or enzymes and its action is specific. It splits up egg-white and no other proteid. It is called into existence by the introduction of egg-white or its haptophor into the circulation of the animal. The introduction of this foreign proteid into the body calls for some means for its disposal. The body has no agent by which this can be accomplished, and, as a consequence, certain cells are called upon to produce such an agent. These cells respond to this call and begin to elaborate the needed enzyme. Gradually and somewhat slowly these cells acquire this new function. By means of this new enzyme the foreign proteid is split up and some of the split products probably serve certain cells in the body as food material, while others constitute a menace to health and even to life. Our haptophor group contains the sensitizing body, that which calls into existence this new function and leads to the elaboration of the new proteolytic enzyme. Our toxophor group contains the substance that endangers the life of the animal. As our experiments have shown, danger to the life of the animal depends not so much upon the amount of the free toxophor introduced into the animal as upon the amount set free at one time. When we first introduce egg-white or other foreign proteid it is slowly broken up and no recognizable harm is done the animal. The same is true when frequent injections are made at short intervals of time. But when this new function of splitting up a foreign proteid is called into existence and time enough is given for the cells concerned in the development of this new enzyme to store this up within themselves, where it probably exists not as an active enzyme, but as a zymogen, then a second portion of this same foreign proteid is introduced, the specific zymogen becomes an active enzyme, and the foreign proteid is split up so rapidly that enough of the toxophor body is set free within a given time to affect seriously the animal and possibly destroy its life.

At first we inclined to the opinion that the interval of 10 or 12 days that must elapse between the administration of the first dose and the development of the condition of full sensitization was due to some temporary immunity induced by the toxophor; but when we found that in the same time and no sooner the same degree of susceptibility could be established by using as the sensitizing agent our haptophor, instead of the unbroken egg-white, we were compelled to abandon this theory. Certainly this would be necessary unless we could show that our haptophor produces by itself an antibody to our toxophor, and after we had demonstrated that this does not happen we were compelled to conclude that neither our toxophor nor an antibody to it has anything to do with the production of the condition of sensitization, not even any influence upon the time period necessary to develop it.

It can easily be understood of what great benefit it is to the animal body to break up a foreign proteid with fulminating rapidity as soon as it gains access to the tissues of the animal, provided that the foreign proteid is a living one. And the only foreign proteids that find their way into the blood of the living animal under natural conditions are living proteids. Unless the body be possessed of the power of quickly breaking up the living proteid, the invader multiplies, feeds upon the proteids of the animal body, converting them into foreign proteids, and finally destroys the host by virtue of the strength gained from its host.

THE CORRELATION OF THE WORK ON EGG-WHITE WITH PREVIOUS WORK ON OTHER PROTEIDS

We have by the same agents in the retort split proteids of diverse origin into poisonous and non-poisonous portions. Some of these proteids are bacterial, others vegetable, and still others are animal. We think it probable that all proteids are made up of poisonous and non-poisonous groups, and that these are combined in the same molecule, much as an acid and a base combine to form a salt. Indeed, we are inclined to hold that a proteid is a highly complex body containing an acid and a basic group, each of which is still complex and of such a character that for the present we must regard each as a proteid in itself. The acid part of the proteid molecule is an active poison when detached from its basic part and injected in this uncom-

bined state into the animal. It acts in the same way when it is separated from its base in the animal body. The acid group owes its poisonous action to the avidity with which it combines with the basic constituents of some of the cellular proteids of the animal body. From the symptoms that follow the injection of the free toxophor into the animal body or the injection of the unbroken proteid into the body of a sensitized animal we infer that the poisonous group endangers the life of the animal by its ready combination with the basic constituents of the proteids of the cells in the respiratory center. The poisonous group, whatever may be the source of the proteid from which it is obtained, induces in the animal the same train of symptoms and death is due to the same cause—to failure of respiration. The acid character of the poisonous group is indicated by the fact that it is best split off from the whole proteid by alkalis and this indication of the acid nature is confirmed by the fact, so well demonstrated in one of the earlier papers from this laboratory, that it combines with alkalis, and when thus combined its poisonous action is reduced.

In another paper we have stated our reason for believing that life is molecular or at least that metabolism, the one essential phenomenon of life, is intramolecular and that every proteid molecule has its chemical nucleus or center of chemical energy and that the poisonous group constitutes this chemical nucleus. The chemical nucleus of each proteid differs in some respects from that of every other proteid and yet all resemble each other in the readiness with which they combine with other bodies and construct whole proteids. In the free state they may be regarded as acid radicals with unsatisfied valences, and this accounts for their fulminating action as poisons when introduced into the animal body in the free state or when set free in the body of the sensitized animal. They act as poisons by tearing off from other proteids their secondary or basic constituents, probably not in whole, but only in part. This interrupts the function of the cell of which the injured proteid in an essential part, and if this function be one, the continued operation of which is essential to the life of the animal, this interruption endangers life and may cause death. The injured proteid in the animal cell is not destroyed; it is only crippled, and if not too suddenly injured it repairs its injury and the animal

lives, although death may have been imminent. In this way we explain the immense importance of the rapidity with which the poison enters the circulation or the rate at which it is set free in the sensitized animal.

Turning now to the non-poisonous or haptophor or sensitizing or immunizing substance, we have found that previous treatment of animals with this portion of the proteid of the colon or of the typhoid bacillus gives specific immunity to the living organism, and that previous treatment with this portion of the egg-white proteid renders the animal susceptible to the next dose of the same proteid, so that it kills. In one instance the life of the animal is saved; in the other it is jeopardized, and in the majority of instances lost. These phenomena seem to be antipodal and yet if we interpret them aright they are identical. The colon or the typhoid bacillus is only a specific, living proteid. It is a proteid in an active, metabolic state, capable of absorbing, assimilating, and multiplying. The egg-white is the product of life and with the potentiality of again becoming a living proteid. The bacillus is made up of labile molecules, while the molecules of the egg-white have passed into a more stable condition. The one is in an active, the other in a resting state; the one is actively engaged in trading in energy, the other is temporarily at least quiescent, and yet both are proteids, markedly similar in their chemical composition and yet characterized by a specific difference. Both are essentially proteids, made up of an acid or poisonous chemical nucleus and a basic or non-poisonous group. The former in its effect upon animals is the same, whether derived from the bacillus or the egg-white, and the latter in the one instance induces specific immunity and in the other specific susceptibility. But the immunity and the susceptibility each consists in developing in the animal body the capability of splitting up a specific proteid. If the living proteid be split up before it has had time to multiply sufficiently to furnish a fatal quantity of the toxophor the animal lives and we say that it has been immunized. If the stable proteid be introduced into the animal body it develops a specific proteolytic ferment, and if enough of it to supply a fatal dose be injected after this function has been edveloped, the animal dies. The first or sensitizing dose of egg-white injected into the animal is split up or digested just as surely as is the second

dose, but the process proceeds so slowly that we can see no effects, and we say that the egg-white is without effect upon guinea-pigs when injected subcutaneously or intra-abdominally. But subsequent injections show how erroneous this conclusion is. The first dose of egg-white has in truth affected the animal profoundly, so profoundly that the effect not only persists in the individual for months and possibly for years, but may be transmitted to the next generation. We do not say that the animal is sensitized, unless some immediate and striking effect follows our treatment; but it seems to us that this view is not altogether correct and that it needs some modification. As we have repeatedly stated the immediate effect, especially a fatal issue, depends first of all upon the rate at which the proteid is split up in the animal, and this depends first of all upon the amount of available proteolytic ferment or rather zymogen stored up in the animal body at the time of the injection. It is probably true that at the time of the first injection there is none of this specific ferment stored in the animal body. It is hardly believable that any cells have preformed in them, even in infinitesimally small quantities, all the specific proteolytic zymogens ready for any emergency. But the foreign proteid must be disposed of, and this is accomplished by the development of a specific ferment. This ferment is developed slowly and digests the proteid of the first dose so slowly that the well-being of the animal is not visibly affected. If now time enough be allowed for the accumulation of a large amount of this specific zymogen in the body-cells and then a second dose of the same proteid be injected, it is split up into its poisonous and non-poisonous constituents and the former induces the symptoms and may cause death. It seems to us that this satisfactorily explains most, if not all, of the phenomena observed in the study of the sensitization of animals to proteids. It has been noted by others as well as by ourselves that when the sensitizing dose is a large one (10 c.c. of egg-white) the animal, after a short interval (10 to 20 days) is not so thoroughly sensitized as it is when the sensitizing dose is smaller (1 c.c.). For corroboration of this compare Nos. 9, 13, and 21 in Table 3 with Nos. 235, and 355 in the same table; but when the time interval runs up into months, the larger sensitizing dose is quite as effective as the smaller one. However, there are so many factors that must be taken into consideration, and inas-

much as one of these, the individuality of the animal, cannot be measured, we must be careful about drawing conclusions; but we have no doubt that a wider experimental observation will give us a correct explanation. At present we are justified in tentatively suggesting the explanation that it takes the animal a longer time to dispose of a sensitizing dose of 10 c.c. than one of only one-tenth this amount. The explanation of the observed fact, that in animals to which multiple injections have been given the time interval between treatments is of marked importance, will be evident to all who have followed us thus far.

We wish to call attention to the fact that in the development of either sensitization or immunity each proteid apparently has its own individuality and characteristics. The haptophor of the proteid of the colon or typhoid bacillus, provided the dose be a small one, will sensitize a guinea-pig within 30 minutes, so that it will successfully dispose of from four to six times the minimum lethal dose of a living culture of the bacterium; but this sensitization begins to diminish in from three to seven days, and is wholly lost in between 30 and 40 days; while the haptophor of egg-white requires from 10 to 12 days to develop a recognizable degree of sensitization. But the condition having been established, it continues, without apparent diminution for months, possibly for years, and may be transmitted to the offspring of the sensitized animal. We infer from this that the haptophor of the bacterial proteid causes only a slight and temporary modification in the animal cells that supply its specific proteolytic enzymes, while the haptophor of the egg-white produces a profound and lasting effect upon the same animal cells. This is also shown by the fact that the bacterial proteid kills at the first dose, while the egg-white must be given a second time. Each proteid will need to be investigated individually before we can confidently make any statement concerning the amount of it necessary to sensitize, the time that must elapse before the condition of sensitization is established, and the continuance of the condition, and we wish to state that while our method of splitting up proteids in the retort has, in the case of all proteids with which we have worked, yielded a toxophor body, it has with several proteids failed to give a sensitizing or immunizing haptophor. The pneumococcus when broken up with alcoholic alkali gives a poison,

but the non-poisonous portion gives no immunity to the living organism, and so far we have not been able to obtain a sensitizing or immunizing haptophor from casein. This is not at all strange, because casein, as is well known, is quite a different proteid structurally from egg-white. It is probable that with other reagents, or with the same reagents in different proportions, we may get from the cellular substance of the pneumococcus and from casein immunizing and sensitizing proteids, but we wish to state clearly that each proteid will need to be investigated before anything can be predicted concerning the haptophor, and it is possible that there may be proteids that contain no true haptophor groups. We make no claim of the discovery of a universal law.

In conclusion we wish to state briefly the deductions which we have drawn from our work as outlined in this and previous papers.

1. All the proteids with which we have worked may be split up into poisonous and non-poisonous portions.

2. The poisonous group is an essential constituent of all the proteid molecules; and while it is not identical, so far as its chemical structure is concerned, in any two proteids, it is similar in its psysiological action in all the proteids which we have investigated.

3. The poisonous group is the chemical nucleus, i. e., the center of chemism or chemical energy, in the proteid molecule.[21]

4. This group must for the present be regarded as a proteid body, the chemical structure of which remains unknown.

5. It owes its poisonous action to the avidity with which it combines with certain groups in the molecules that constitute the cells of the respiratory center; in other words, it is a respiratory poison.

6. The exact effect of the poisonous group upon an animal depends upon the manner and the rate of its introduction into the body. It may induce acute, subacute, or chronic poisoning.

7. Life is molecular, or metabolism, the essential phenomenon of life, is intramolecular. A bacterium consists essentially of a living proteid, made up of labile molecules that are constantly reacting with outside matter, thus growing and multiplying. The pathogenicity of a bacterium depends upon its ability to multiply in the animal body and convert certain constituents of the animal body into a foreign proteid, and when the foreign proteid thus formed is broken

up in the body of the sensitized animal, its poisonous group is set free and induces the symptoms of disease and death.

8. Proteid susceptibility and immunity are different manifestations of one and the same process. Both depend upon the development in the animal body of a specific proteolytic ferment. When this specific ferment splits up a living foreign proteid before it has time to multiply we say that the animal is immune. When this cleavage action is less prompt, but sufficiently so to split up the living proteid before it elaborates a fatal amount of the poison, the animal sickens, but recovers. When the action of the ferment is still less prompt and the living proteid constructs enough poison to kill, then its liberation causes death. When this specific proteolytic ferment has been developed in the animal by previous treatment with a dead or stable proteid, it is easy to inject a small dose of the same proteid in sufficient quantity to quickly induce symptoms and to kill, then we say that the animal has been sensitized or is in a condition of hypersensibility. With a dead, stable proteid it is easy so to adjust the dose that the animal will show no symptoms, or manifest the first and second stages and recover, or die. With a live or labile proteid the conditions are much more complicated and consequently the result is more uncertain and less controllable.

9. Life is rythmic, i. e., the living, labile molecule reacts with outside matter rythmically, but the *tempo* of this reaction is subject to change when conditions are altered. One strain of the pneumococcus may kill a guinea-pig in a dose of one-millionth of a c.c., while 1 c.c. of another strain may be required to produce the same result. This difference in virulence is, we believe, due to difference in the *tempo* of the molecular reactions, and this introduces into certain cases of infection a variable which at present we have no means of measuring or controlling.[20]

10. In some, not in all, of the proteids with which we have worked the non-poisonous portion has proved to be an immunizing or sensitizing haptophor, giving a specific immunity to its own living, labile proteid, or developing a specific sensitization to its own dead, stabile proteid.

11. These specific haptophors do not sensitize animals to themselves because they contain no poisonous group.

12. Sensitization consists in developing in the animal a specific proteolytic ferment which acts upon the proteid that brings it into existence, and on no other. It may be interesting to state here that we have demonstrated that guinea-pigs sensitized with woman's milk respond to a second treatment with woman's milk and not to cow's milk, and those sensitized to cow's milk do not react with woman's milk.

13. This specific proteolytic ferment stored up in the cells of the animal as a result of the first treatment with the proteid remains in the cells as a zymogen until activated by the second injection of the same proteid.

14. Our conception of the development of a specific zymogen supposes a rearrangement of the atomic groups of the proteid molecules of certain cells in the animal body, or an alteration of the molecular structure. In other words, we regard the production of the specific zymogen not as the formation of a new body, but as resulting from an alteration in the atomic arrangement within the proteid molecule and a consequent change in its chemism.

15. Some proteids, in developing the specific zymogen, produce profound and lasting changes in molecular structure, while the alterations induced by others are slighter and of temporary duration, the molecular structure soon returning to its original condition.

16. In order to serve as a good sensitizer the proteid must be in solution. This is the reason why the haptophor of the colon bacillus sensitizes an animal so much more readily than either the live or the dead bacillus. In fact, as we have seen, the haptophor may sensitize the animal within 30 minutes, so that it will resist from four to six times the minimum or the usual lethal dose of the living bacillus.

17. In treating infected animals the sensitizing haptophor must be employed in minimum doses, only enough to activate the zymogen, because larger quantities consume the activated ferment themselves and thus protect the infecting organism. Ten mg. of the haptophor of the colon bacillus will protect a pig against quantities of the living bacillus (administered 30 minutes later), against which 50 mg. would fail to afford any protection. By minimum doses we do not mean infinitesimally small, and just what they are can be learned only by experiment and experience.

18. What cells are concerned in the elaboration of the specific zymogens we can only surmise at present, but on *a priori* grounds we suppose that they belong to the mesodermal tissue, one of the functions of which, even in the unicellular organism, is to dispose of and utilize proteids of diverse nature.

19. It is possible that studies along this line may be of service in investigating problems of heredity, and substantiate Loeb's statement that heredity is a chemical question.

20. The transmission of the conditions of sensitization seems to be in accord with the view that mutations are not the result of slowly established alterations, but appear suddenly.

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OBSERVATIONS ON NATURAL IMMUNITY AND SUSCEPTIBILITY TO DIPHTHERIA TOXIN.*

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THE RESISTANCE OF THE WHITE RAT.
ARTIFICIAL EXALTATION OF THE WHITE RAT'S NATURAL IMMUNITY.
HAS DIPHTHERIA TOXIN A CUMULATIVE ACTION UPON THE WHITE RAT?
THE FATE OF DIPHTHERIA TOXIN IN THE BODY OF THE WHITE RAT.
THE RESISTANCE OF THE RABBIT.
IMMUNIZATION AND HYPERSUSCEPTIBILITY OF RABBITS.

THE RESISTANCE OF THE WHITE RAT.

ALTHOUGH frequent mention of the high degree of immunity displayed by the rat to diphtheria toxin is to be found in the literature of antitoxic immunity, there have been but two attempts to ascertain its extent. Roux and Yersin[1] in 1888, working with black-and-white rats, observed no effect from the subcutaneous injection of ten times

TABLE 1.

SUBCUTANEOUS M. L. D. OF DIPHTHERIA TOXIN FOR THE WHITE RAT.

Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses† Injected	No. Days Survived
1	113	0.0348	10	3.00	10	‡
2	137	0.42196	10	3.00	100	‡
4	150	2.31	10	3.00	500	‡
5	147	1.176	42	3.00	800	‡
18	108	1.728	45	2.73	1,000	‡
19	132	3.168	45	4.17	1,500	6
38	200	6.45	45	7.45	1,875	9
20	120	3.84	45	4.84	2,000	6
36	140	4.816	45	5.80	2,000	5
9	140	3.60	42	4.10	2,500	5½
31	206	8.838	45	9.83	2,500	6
28	130	6.71	45	7.71	3,000	5
12	177	6.195	42	7.20	3,500	4½
13	166	6.225	42	7.22	3,750	5½
14	120	4.50	42	5.50	3,750	4½
15	157	6.084	42	6.70	3,875	6½
6	144	5.76	42	6.70	4,000	3½
7	142	11.36	42	13.00	8,000	1½

the guinea-pig lethal dose, but pursued the subject no further. Cobbett,[2] some ten years later, using both white and black-and-white

* Received for publication May 5, 1907.

† The word gram-dose is used here to signify one guinea-pig M. L. D., calculated per gm. of the anima under discussion. In other words, one gram-dose is a dose of toxin containing 1/250 of a guinea-pig M. L. D. for each gm. of weight of the animal injected.

‡ Animal survived entirely.

rats endeavored to determine the subcutaneous dose just sufficient to kill in four or five days, and observed that, weight for weight, it was "from 1500–1800 times as great as those which suffice to kill guinea-pigs of 250 grams."

Inasmuch as the experiments planned required a knowledge of the exact minimum lethal dose for white rats, it was thought advisable as a first step to attempt a verification of Cobbett's results and to this end a large series of white rats were injected subcutaneously with varying quantities of diphtheria toxin with the results recorded in Table 1. If death on the fourth day following the injection is taken as the criterion, the data show that, weight for weight, the minimum lethal dose for the white rat averages about 3,500 times as much as for the guinea-pig of 250 gms., a figure considerably higher than that obtained by Cobbett.

This investigator used rats ranging in weight from 75 to 115 gms. and averaging not over 100 gms., while the animals used here were considerably larger, as can be seen from the recorded weights; and although in both cases the dose of toxin was corrected to the weight and calculated per gm. thereof, the discrepancy in the two results may nevertheless be due to this point. Assuming, then, that both Cobbett's results and these are correct for the animals experimented upon, the inference is that the tissues of the grown or half-grown rat are, gram for gram, about twice as resistant to this toxin as those of the quarter-grown rat, such as Cobbett used. Upon testing this hypothesis experimentally, the results from a small series of animals divided into pairs, each consisting of one rat weighing not over 100 gms. and one-half or full-grown animal, each pair being given the same dose of toxin per gm. of body weight, appear to bear out the above (Table 2). While this point is scarcely of much importance,

TABLE 2.

RELATION OF RESISTANCE TO AGE IN THE WHITE RAT.
Subcutaneous Injection of Diphtheria Toxin.

Series No.	Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses Injected	No. Days Survived
I	38	200	6.45	45	7.45	1,875	9
	39	88	2.838	45	3.84	1,875	4½
II	28	130	6.71	45	7.71	3,000	5
	22	95	4.56	45	4.56	3,000	2½

since it is well known that very young animals are more susceptible to infectious diseases, as a rule, than older ones, the topic has been brought into this paper partly to offer an explanation of the discrepancy between the results of Cobbett and those in Table 1, and partly because of the striking manner in which this phenomenon is displayed and the interesting ratio existing between the resistance of the grown and half-grown rat and that of the quarter-grown animal, i. e., about two to one, and the length of the period of survival after the administration of a lethal dose.

In all experiments, unless otherwise indicated, the toxin was introduced beneath the skin of the abdomen by means of a Hitchens[3] reservoir syringe, which insures the injection of all the toxin measured out. To reduce the element of error arising from daily fluctuations in weight, all animals were fed and weighed at definite hours, and the dose given was always calculated per gm. of body weight. When a considerable quantity of toxin was to be injected, as was frequently the case with rats, the syringe containing the toxin was first placed in the thermostat to warm it to the body temperature, in order to lessen the shock to the animal, which even then was considerable, and in some cases so severe as to cause death, after some hours in a peculiar state of helplessness accompanied by an ague-like clonus of the skeletal muscles. While this phenomenon always accompanied the administration of large doses, it is not specific, being occasioned by large quantities of physiological salt solution as well. The toxins employed were accurately-standardized in respect to the standard-weight guinea-pig M. L. D., and were tested monthly for deterioration.

Cobbett[2] states that the tissues of the white rat are little affected by large doses of diphtheria toxin, and that he was unable to find evidence of necrosis. The autopsies performed in the course of these experiments corroborate this. As is well known, the postmortem appearances in guinea-pigs dead of diphtheritic intoxication consist of a fibrinous exudate and ecchymoses at the site of injection, enlarged and hemorrhagic adrenals, and serous exudates into the pleurae. None of these occur in the rat, and, in fact, no lesion was found that could be considered as characteristic of the administration of diphtheria toxin. While, then, no characteristic lesion is apparent, a sublethal dose even as small as 500 times the guinea-pig M. L. D. per

gm. of the rat's weight causes a complete alopecia, which varies in extent from covering a circumscribed area at the injection site to almost the whole ventral surface, according to the quantity of toxin employed, the number of injections, and the interval between them. This lesion cannot be considered absolutely specific to diphtheria toxin, however, as even physiological salt solution may cause it, though to but a slight degree. Thus, to cite one instance, a rat was given 1.7 c.c. of toxin containing one thousand toxic gram-doses plus 1 c.c. of physiological saline solution. Four days later the first stages of the alopecia were observed. On the tenth day it had reached its maximum, covering the entire ventral surface from the inguinal region to the neck, and on the sixteenth day the animal had completely recovered. A second rat which had been given 10 c.c. of physiological salt solution at the same spot developed a small bare patch on the third day which reached its maximum three days later when its dimensions were about 2×0.5 cm. and which disappeared by the tenth day. A third rat given two and one-half times the quantity used for the first rat but of sterile infusion broth, containing no toxin, developed only a slight suggestion of alopecia. While the alopecia following the injection of salt solution or broth is undoubtedly due to traumatism, it seems as though the toxin must have some further action to account for the markedly greater lesion resulting from the injection of a smaller quantity of liquid. Further, the phenomenon occurs to some extent after intraperitoneal as well as subcutaneous injection of toxin but not of salt solution or broth. This alopecia has not been observed in guinea-pigs injected subcutaneously, but has been seen once or twice in rabbits.

From one to three days before death, rats frequently show a peculiar tendency to keep one or both hind-legs strongly flexed. This condition is not a true paralysis, such as is seen in guinea-pigs from the effects of late diphtheritic intoxication, for upon strong stimuli the animal is able to extend the limb. Another point of difference is that here the flexors predominate, while in the guinea-pig it is the extensors. Nevertheless, it may be analogous to that of guinea-pigs in that it is due to the presence of toxone in the toxin, because these pareses were quite common with one toxin but wholly lacking with another.

To determine the minimum lethal dose of diphtheria toxin for rats by intraperitoneal administration, another series was injected in this manner with the results compiled in Table 3, which indicate that the

TABLE 3.

INTRAPERITONEAL M. L. D. OF DIPHTHERIA TOXIN FOR THE WHITE RAT.

Rat No.	Weight Gms.	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses Injected	No. Days Survived
27	142	5.86	45	6.86	2,400	3½
24	153	5.79	45	6.79	2,200	4
25	164	4.98	45	5.98	1,800	3½
45	170	4.386	45	5.38	1,500	3½
49	242	5.9145	46	6.90	1,300	3½
55	155	3.4969	46	4.50	1,200	4
44	115	1.978	45	3.00	1,000	*

*Animal survived entirely.

intraperitoneal M. L. D. is about 1,200 times as great as that for the standard-weight guinea-pig subcutaneously, corrected to the rat's weight, or, to express it more conveniently, is about 1,200 gram-doses.

The great discrepancy between this figure and that for subcutaneous injection (3,500 gram-doses) appears to be due to one of the following three causes: (*a*) In spite of the absence of connective-tissue necrosis at the injection site, the toxin is bound by the connective tissue cells. (*b*) The surplus toxin is held mechanically in the meshes of the connective tissue, the cells of which having no affinities for the toxin are immune to it, and is there either gradually transformed into some inert substance, or, more probably, held for a time and yielded so slowly to the circulation that the amount is insufficient at any one time to cause symptoms of intoxication, since, as will be shown later, the tissues of the rat do not store up small quantities of toxin giving rise to the so-called "cumulative" effect. (*c*) The other possibility is that the surplus toxin is bound by epithelial cells which, in the case of subcutaneous injection, would be those of the hair follicles, sebaceous and sudorific glands, since their anatomical distribution causes them to be the first to come in contact with the toxin.

In favor of the second view, mechanical retention by the connective tissue, is the fact that, as will be shown later, toxin remains in the connective tissue at the injection site for a comparatively long time, while the third hypothesis, epithelial binding, is supported by the fact that alopecia develops in areas, as around the axillae and neck, where

it can be shown no toxin is held in the subjacent connective tissue. Indeed, the marked alopecia following subcutaneous injection of toxin is one of the strongest points in favor of the third theory. To be sure, physiological salt solution likewise produces an alopecia, but one very restricted in extent, and it is but reasonable to expect that the local traumatism resulting from the injection of 8 or 10 c.c. of liquid would suffice to interfere with the nutrition of the epithelial elements at the site and cause their atrophy. That something more than this is concerned in the case of toxin injections is shown by the far greater extent of the alopecia resulting from injection of toxin consisting of but a fraction of the control volume, and which even reaches, as stated above, to areas where the connective tissue contains no demonstrable toxin. On the other hand, however, is to be mentioned the occurrence of abdominal alopecia following intraperitoneal injections of toxin, and its appearance after the injection of a sublethal intraperitoneal dose shows it cannot be the chief agency to which is due the difference between the subcutaneous and the intraperitoneal lethal doses. In this case probably but a trifling amount of toxin brought there by the blood stream damages the hair follicles sufficiently to cause the alopecia. Why it appears only at or around the point where the needle was introduced is, however, not clear.

ARTIFICIAL EXALTATION OF THE WHITE RAT'S IMMUNITY TO DIPHTHERIA TOXIN.

Kuprianow[4] succeeded in obtaining an artificial antimicrobic immunity to diphtheria bacilli in gray rats, due to the formation of antibodies which he found in the serum, but the exaltation of the natural antitoxic immunity of the rat has been attempted previously only by Behring,[5] who failed in spite of the most careful increase from small to larger doses.

To test the possibility of producing an artificial increase in the rat's immunity, an animal was given a subcutaneous injection of 500 gram-doses of diphtheria toxin which was followed at intervals of a week or 10 days, when the animal's weight had returned to normal, by doses of 1,000, 2,000, and then 4,000 gram-doses making four injections altogether. The animal died seven and one-half days after the administration of the last dose. As the last dose was somewhat in

excess of the rat M. L. D. which kills in four days as determined above, the period of survival in this case may be held to indicate either individual variation, or the acquisition of a small degree of additional resistance to the toxin. A second animal was then started with approximately weekly injections of 500, 1,000, 2,000, 2,800, 3,400, 3,800, 4,000, 4,200, 4,500, and 5,000 gram-doses in the above succession. It died one week after the administration of the 5,000 gram-doses dose from shock following the injection of 5,800 gram-doses. In this experiment the results are unequivocal and the acquisition of additional immunity is evident. During its course the animal gained about 18 per cent in weight, and finally withstood the injection of nearly 50 per cent more than the four-day fatal dose with no appreciable signs of disturbance, the weight remaining practically stationary during the succeeding week. As the animal's death was due, not to intoxication from the last dose of 5,800 gram-doses, but merely to the shock from the injection of so large a quantity of liquid (about 11 per cent of the body weight), it is impossible to say to what degree this artificial immunity might be raised. Apparently the only check on an indefinite increase by means of small gradations as above is the volume of liquid that can be introduced subcutaneously without producing fatal shock. Experiments are at present under way to test this hypothesis as well as to see if the serum of an animal, immunized in this manner, possesses any antitoxic powers.

It is interesting to note that the artificial immunity thus acquired is transitory in nature and persists less than one month if injections are withheld.

HAS DIPHTHERIA TOXIN A CUMULATIVE ACTION TOWARD THE WHITE RAT?

Certain drugs like mercury, lead, the iodides, and arsenic, as is well known, if administered in small doses over a long period, have a much more strongly poisonous effect than if the sum of all these doses be given at once. This is held to be due to an accumulation in the tissues of a fraction of each dose administered which eventually has increased to the lethal quantity, while from a single large dose (e. g., lead) not enough is absorbed to produce symptoms. The exhibition of such a cumulative action by diphtheria toxin upon the

rat would help to explain the latter's high resistance toward this toxin, as well as show whether the toxin can be stored up in the tissues of the rat if given in sufficiently small doses. To determine this point the following experiment was performed:

A rat was given eleven injections of 1,000 gram-doses, five injections of 2,000 gram-doses, and then one injection of 3,000 gram-doses; whereupon the experiment was interrupted by the pregnancy of the animal. The rat had nevertheless received, in the course of about two and one-half months $\frac{24,000}{3,500}$, or approximately seven times the lethal dose, and instead of showing any ill effects from the treatment, gained weight, carried through a successful pregnancy, and even acquired some degree of artificial immunity, as was shown by the progressively smaller disturbance following the injections, although the latter were twice increased. Further experimental evidence of the absence of cumulative action on the rat by this toxin is afforded by the immunization experiments described above.

The absence in the rat of the phenomenon of hypersusceptibility displayed by the guinea-pig after the injection of a number of small doses is demonstrated by another experiment. A rat was given 38 daily injections of 100 gram-doses, or $\frac{1}{3}\frac{8}{5}$ of the rat M. L. D. each, on five days in each week, so that at the end of the eighth week the animal had received $\frac{3}{3}\frac{8}{5}$ M. L. D., or somewhat more than the fatal dose, but distributed over two months. The fact that the rat lived and even gained weight during the experiment shows that hypersusceptibility is not concerned in the reactions of the rat to diphtheria toxin.

THE FATE OF DIPHTHERIA TOXIN IN THE BODY OF THE WHITE RAT.

The advances that have been made along the line of antitoxic immunity have been accomplished almost wholly through study of the artificial type thereof. That a clearer conception of natural immunity would go far toward the solution of the problems of antitoxic immunity in general will scarcely be disputed, but up to the present time very little work has been done in this field. While a few researches have been published dealing with the fate of tetanus toxin in certain of the lower forms, hardly anything has been done in regard to the mammals, notwithstanding the prime importance of a knowledge of the mammalian reactions. In the hope of throwing a little light upon certain

phases of mammalian natural antitoxic immunity, the following experiments were undertaken.

Roux and Borrel[6] have shown that, in spite of the high resistance of the rat to diphtheria toxin, the animal is extremely susceptible when the toxin is injected intracerebrally, 0.1 c.c. producing death in two or three days; hence the natural immunity displayed toward other modes of injection must rest upon the failure of the toxin to reach the brain. Behring[5] supposed that this was due to a "special property of the cell-free blood," i. e., antitoxic principles in the serum; while Cobbett[2] states that the serum of white rats in quantities of 1 c.c. does not protect guinea-pigs from a little over the minimum lethal dose of toxin. The two views are thus diametrically opposed. In order to determine this point, a guinea-pig of test weight was injected with a mixture of the M. L. D. of toxin and 0.6 c.c. of normal rat serum. The latter afforded not the least protection. As it is possible that antitoxin does appear in the rat's blood but is generated only by the stimulus afforded by the presence of toxin, a second rat was injected with 800 toxin gram-doses subcutaneously, and the above experiment repeated after the lapse of a week, with the same result. The objection may be raised here that inasmuch as 800 gram-doses are less than the difference between the subcutaneous and the intraperitoneal rat M. L. D.'s and therefore within the limits of the amount of toxin taken up by the connective tissue or epithelial structures at the injection site, too small a proportion of the body cells are exposed to the stimulus afforded by proximity of toxin, and thus only an inappreciable amount of antitoxin is formed and finds its way into the blood stream. While this objection has some weight, still the toxin if not actually bound chemically by cells at the site must be eliminated some time, and if it is yielded to the circulation slowly and in small quantities, the long continued exposure to it of all the body cells ought to afford excellent opportunities for antitoxin formation. An experiment was performed, however, to settle the question by examining the serum of a rat subjected to intraperitoneal administration of a sublethal dose (1,000 gram-doses). It was found that 1 c.c. of such serum two weeks after injection afforded no protection, so that we are justified in concluding that the rat's immunity is not due to circulating antitoxins. The presence of antitoxin in the blood of rats which have

been actively immunized against the toxin is, of course, another question and has no bearing, strictly speaking, upon natural immunity. Behring bases his belief that the immunity of rats is due to antitoxin in the blood upon an experiment in which he injected large doses of diphtheria toxin into rats, intraperitoneally, and then after three hours bled them and injected 4 c.c. of their serum into guinea-pigs, also intraperitoneally. The latter showed no signs of intoxication, although the same quantity of serum of animals susceptible to this toxin gives distinct signs of intoxication in guinea-pigs, the same procedure being observed; and the blood of immunized guinea-pigs gives the same results as that of normal rats. The flaw in this reasoning is obvious. Absence of toxin in the blood three hours after injection is, at least, just as probably due to its failure to get into the blood stream at all, as to the presence of specific antibodies there to neutralize it.

The next step was to attempt to follow the toxin throughout its sojourn in the rat's body from the time of injection until its elimination in the urine, which Cobbett[7] has shown to take place. The only other work of this nature upon a naturally immune animal is the experiment of Brunner[8] on dogs, in which he tested the blood and a compound emulsion of the liver, spleen, kidneys, and muscle of an animal killed after a toxin administration, by injection into other dogs. The blood, given intravenously, yielded negative results, while the tissue emulsion, given intraperitoneally, killed in 18 hours.

Although the literature upon the fate of diphtheria toxin in immune animals is so scanty, there are a few researches in regard to the fate of tetanus toxin in some of the lower animals that are naturally immune to it, and for purposes of comparison it may be worth while to cite them briefly.

Asakawa[9] and Vaillard[10] found that tetanus toxin persists for five or six days in the blood of the chicken. The latter has shown further that the sexual glands alone of all the viscera absorb some of the toxin; no antitoxins occur in the blood. Metchnikoff[11] has proven that this persistence in the blood is due to the absorption of a portion of the toxin by the leucocytes. In regard to the poikilothermous species it is reported by the same investigator[12] that in the alligator the toxin is rapidly eliminated from the blood even when kept at relatively low temperatures (20° C.) though in the marsh-turtle the toxin passes quickly into the blood and remains localized there for more than four months. Two months after injection of 500 mouse M. L. D.'s the blood of a green lizard kept at 20° was still extremely toxic while in

the scorpion the toxin remains in the blood for but a few days passing thence to the liver only and remaining there unaltered for a month or more. Green frogs, again maintain the toxin in their blood for at least two months.

To trace diphtheria toxin through the rat's body, a series of guinea-pigs were given subcutaneous injections of suspensions of the various tissues of rats killed at different intervals after the administration of a sublethal dose of toxin (3,000 gram-doses) subcutaneously.

The suspensions were prepared by grinding up the tissue with physiological salt solution in a sterile mortar, straining through cheese-cloth to get rid of the reticulum and finally diluting with salt solution up to 4 c.c. The amount of each tissue used was not weighed each time but as close an approximation as possible to the same volume of a tissue was used in each experiment. Of the blood 1.25 c.c. were injected. This quantity would contain about 15 guinea-pig M. L. D. if the toxin were uniformly distributed throughout the rat's body. Of the other tissues about 1.7 gm. of brain (entire viscus) were injected, which would have a toxin equivalent of 21 M. L. D.; of connective tissue 0.25 gm. (3 M. L. D.); of kidney (one viscus) 0.7 gm. (8.5 M. L. D.); of muscle (right and left pectoralis major) 2 gm. (24 M. L. D.); of liver 1 gm. (12 M. L. D.); of spleen 1 gm. (12 M. L. D.); and of adrenals (both) 0.045.gm. (0.75 M. L. D.). Inasmuch as the injection of normal rat tissues gives rise to abscesses and eventually ulceration in guinea-pigs, whereby fatal infections were often incurred, acute death within the arbitrary limit of one week was taken as evidence of the presence of toxin while survival beyond that period was held to indicate its absence in the tissue examined. It may be mentioned here that this division was easily made as there were no deaths in the entire experiment between the sixth and the fifteenth day after injection. In all instances the typical postmortem picture of diphtheritic intoxication was displayed by the animals dying during the first week. In rather less than half the acute cases the gastric or duodenal hemorrhages of Rosenau and Anderson[13] were observed. In the 18-hour series in addition to the routine tissues tested and recorded, the lungs, thymus, myocardium, lymph glands, and stomach wall were also tried, with negative results.

Upon referring to Table 4 the history of the toxin injected subcu-

TABLE 4.

LOCATION OF DIPHTHERIA TOXIN IN BODY OF WHITE RAT AT VARIOUS INTERVALS AFTER INJECTION.

TISSUE	NO. HOURS AFTER INJECTION							NORMAL
	18	24	48	72	96	120	168	
Adrenals	−	−	−	−	−	−	−	−
Blood	+	+	+	−	−	−	−	−
Brain	−	−	−	−	−	−	−	−
Connective tissue (site)	+	+	+	+	+	+	+	−
Connective tissue (axillae and neck)	−	+	+	−	−	−	−	−
Kidney	−	−	+	+	−	−	−	−
Liver	+	+	+	+	+	−	−	−
Muscle	−	+	+	−	−	−	−	−
Spleen	−	−	−	−	−	−	−	−

taneously is readily traced. Eighteen hours after injection part of

the toxin has found its way from the connective tissue interstices at the injection site to the blood stream, and has been taken up from the latter by the liver alone of the viscera. Six hours later it appears in appreciable quantity in the general connective tissue of the body and in the skeletal muscles, while 24 hours after this it is to be found most generally diffused over the body, the brain and spleen and adrenals alone being free from it. In another 24 hours it has disappeared from the blood, muscle, and general connective tissues, and on the following day the kidney fails to show the toxin. On the fifth day after the injection the toxin abruptly disappears everywhere except from the original injection site, where it can be detected for seven days altogether.

Thus when a sublethal dose is injected under the skin of the rat, a part remains unchanged in the connective tissue at the injection site for a comparatively long period. The remainder soon gets into the blood stream, undoubtedly by way of the lymphatics. From the blood it is gradually taken up by the various tissues until 48 hours after the injection when the toxin is diffused generally over the body. After this period a receding from these organs and tissues occurs, until on the fifth day it can only be detected at the original site. It is interesting to note that after the period of maximum diffusion (48 hours), the toxin disappears from the blood though persisting in other tissues. It suggests that the accumulation of toxin in the connective tissue at the site is not dissipated by being surrendered gradually to the circulation and thence excreted by the kidney, as is the ordinary physiological procedure, or by the liver, as is the usual fate of toxic substances, but that it is more probable we have to do here with a rather considerable discharge into the circulation of the toxin in excess of what can be accommodated by the local connective tissue. The surrendered portion circulates in the blood, and, being brought into intimate relation with the various tissues, is taken up by them. This is supported by the results of a similar series of experiments in which the toxin, 1,100 gram-doses, was introduced intraperitoneally so as to eliminate the influence of the connective tissue. After 24 hours the toxin could be found in the liver alone of all the tissues, and it had disappeared thence after 48 hours. The rapidity of the disappearance of the toxin in this case is undoubtedly due to its being surrendered to the blood

far more rapidly by the peritoneum than by the subcutaneous tissue. This would explain the disappearance of the toxin from the blood after the period of maximum diffusion in the body, when it might be supposed that all the toxin in the blood is removed from it by the other tissues, but it complicates the question of elimination. As mentioned above it has been shown that diphtheria toxin is excreted in the urine of the rat, at least a few hours subsequent to injection. It is easily seen how a considerable portion of the toxin can thus be eliminated during the period in which the toxin is to be found in the blood. But what is the mechanism in regard to the elimination of that portion remaining in certain tissues after the blood ceases to contain toxin? Is it yielded to the blood and thence to the kidneys so gradually and in such small quantities that even so delicate an indicator as the guinea-pig biological test is insensitive to it, though huge doses of toxin are given the rat; or do the tissues in which it is held break it down or build it up into an inert substance? The absence of antitoxin in the rat's blood by no means precludes the possibility of an intracellular neutralization, although at present we have no definite evidence of such an occurrence. If the former hypothesis of gradual elimination be true, it might be possible to get experimental evidence by injecting a guinea-pig with a large quantity of rat's blood in the hope that if only a small quantity of toxin be in the blood, the amount of blood used would bring this toxin modicum up to the guinea-pig M. L. D. Of course while a positive result would prove the case, a negative result could throw no light upon the subject either way, for the amount of toxin, if present, might be sublethal even in the larger quantity of blood. The latter was the fate of an experiment of this kind. A guinea-pig was injected with 6 c.c. (about five times the quantity usually used in these experiments and which would contain about 75 guinea-pig M. L. D. if the toxin were uniformly distributed) of the blood of a rat killed 96 hours after the toxin was injected. This period was chosen because then the process should be most marked since at this time the elimination of toxin from the various tissues is at its height. The animal survived the test period, living three weeks, and thus giving no information. Larger quantities of blood than 6 c.c. were not employed because the injurious effect (ulceration) of normal rat's blood itself is so pronounced as to invalidate conclusions.

Regarding the other tissues it might appear that the toxicity of suspensions of a such highly vascular organ as the liver for example, is due merely to the amount of blood it happened to contain at the time. While this is probably the case to a considerable extent, and particularly true of the liver in the earliest stages, the fact that the toxin is evident there some time after disappearing from the blood shows that the cells themselves play an important rôle, and that, at least in the later stages, it is the tissue and not its blood content that gives the reaction. The fact that the liver contains toxin at all periods up to the fifth day, and is the only tissue (site excepted) so doing, might be regarded as a hint that it has something to do with the transformation or elimination of toxin. But if so, how does the toxin taken up by other tissues reach the liver after its disappearance from the blood?

As for the kidney, the absence of toxin there before the second day apparently does not agree with Cobbett's statement that the toxin is eliminated by the urine, but the small amount of the latter present in the kidney is insufficient to cause symptoms even when it contains toxin, while the results for the second and third day show that even by that time the kidney tissue itself has absorbed some toxin from its blood-supply. The results with the spleen, showing the absence of demonstrable toxin at all stages, are difficult to explain in view of the great vascularity and enormous blood content in proportion to its own weight, as it would seem that this alone would cause intoxication. Of course in testing the blood a larger quantity was employed than was contained in the bit of spleen, and it is possible that this latter amount was just too small to contain a fatal dose.

The direct or "in vitro" power of the spleen, as well as of the myocardium, liver and brain, to neutralize toxin was tested* after the method of Wassermann and Takaki.[14] It was found that these tissues were unable to bind even two M. L. D. in such a manner as to protect a guinea-pig injected with the triturated emulsion, although even with a uniform distribution of the toxin throughout the rat's body each gram of body tissue should be capable of neutralizing about 12 M. L. D., as indicated above. The effect of these tissue-triturate

* In the experiments testing the effect of mixtures of tissue emulsions and toxin to determine the existence of an "in vitro" neutralizing power by these viscera I was assisted by Mr. C. D. Enfield, a student in this laboratory.

emulsions and toxin was the same whether the tissue was ground with the toxin or the expressed fluid mixed with the toxin after grinding, and whether the mixture was kept at 20° C. or 37° C. for 30 minutes previous to injection. It is evident therefore that the living cells and not merely the tissue substance are necessary for the absorption of the toxin. These living cells do not include the leucocytes which, it will be remembered, were found by Metchnikoff to take up tetanus toxin in the fowl. The point is proven by the following experiment:

A normal rat was bled into a sterile 1 per cent solution of sodium citrate in 0.85 per cent sodium chloride solution to prevent coagulation. The mixture was centrifugalized and washed twice in sterile physiological salt solution to remove traces of citrate, the supernatant liquid removed and 1.5 c.c. of the upper layer of corpuscles taken as leucocytes. To these were added 2 M. L. D. of toxin and enough normal salt solution to make the whole up to 4 c.c. This was incubated at 37° for 30 minutes and then injected into a guinea-pig. No protection was afforded. Since each gram of leucocytes would receive about 12 M. L. D. if the 3,000 gram-doses of toxin used in the above experiments had been uniformly distributed throughout the rat's body, and since they would be able to accommodate many times that quantity if the leucocytes are the real protective agency as in the case of the fowl, failure to protect against 2 M. L. D. indicates that the leucocytes are not to be considered in the resistance mechanism.

Besides the spleen, the brain is the only tissue which at no stage gives evidence of containing toxin. The subject is simpler here than in the case of the spleen, for the rat's extreme susceptibility to intracerebral injection of diphtheria toxin, shows that if any were to reach the brain the animal would quickly succumb. Indeed the entire problem of explaining the immunity of the white rat to the subcutaneous injection of toxin is expressed in the question why the toxin does not reach the susceptible central nervous system. The old theory of Ehrlich's school, that natural immunity is due to a scarcity of suitable receptors to bind the toxin, is quite inadequate, for (*a*) if there are no receptors capable of combining with the toxin, it would not be possible to kill the animal, no matter what the dose used, and (*b*) if there were but few suitable receptors it would require a smaller dose to saturate these and produce death than in the case of a susceptible animal which possesses a larger number of receptors. Similarly it cannot be due merely to the presence of an unusually large number of receptors in the connective tissue at the injection site which absorb all the toxin and leave only a small fraction, or none at all, to get into the blood stream, for these ex-

periments show that toxin does get into the blood in considerable quantity, and, indeed, the results of experiments with the viscera of rats injected intraperitoneally bear out the above completely. Lastly, the immunity of the rat cannot be due to the absorption of the toxin by the leucocytes, as Metchnikoff has shown takes place in the fowl, because (*a*) the toxin persists in several tissues after having disappeared from the blood, (*b*) the pre-eminently leucocytic tissues such as the lymph glands and the spleen show no evidence of the presence of toxin, and (*c*) "in vitro" experiments with living leucocytes show them to lack the power of taking up toxin. To be sure, the lymph glands were examined only at the 18-hour stage, but the spleen was tested at each period, and further, if the leucocytes did have the same function as in the fowl, it should have manifested itself in the lymph glands at the time they were examined. The subject is thus exceedingly complex, and inasmuch as the data obtained contradict our present hypotheses, it is, perhaps, permissible to attempt to formulate a theory to explain natural immunity in the rat, basing it upon the information secured in the foregoing experiments. The hypothesis I beg to submit is that there are three types of cells in the rat in relation to diphtheria toxin, (1) those possessing no receptors capable of uniting with diphtheria toxin, and hence inert in respect to it; (2) those possessing receptors capable of uniting with it, but whose receptors (a receptors) have but a low degree of affinity for the toxin; when the latter is bound, however, the toxin enters into such a combination with the protoplasm of the cell that its vitality is destroyed. The best examples of this type are the brain cells. (3) The third type of cell also possesses receptors able to bind the toxin, but these (β receptors) differ from the a receptors of the nervous elements in that they have a high degree of affinity for the toxin molecule. Another point of difference between the cells of the second and third types is that when bound by the receptors of the latter, the toxin molecule does not enter into a combination with the cell protoplasm, which is incompatible with continued life of the latter, but is treated by the cell as a food, and after remaining in the cell a sufficient length of time is broken down to simpler bodies through the agency of intracellular enzymes, perhaps, whose function it is so to treat the various food elements taken up by the cell. Eventually part of the original toxin molecule

is built up into the protoplasm of the cell, while the remainder, of course no longer to be considered as a toxin, is eliminated by the cell in the usual manner. Such a body could not be detected in the blood. Examples of cells of this last type are those of the liver, and kidneys.

Turning now to Table 4, how is it to be interpreted according to the foregoing hypothesis? When the toxin is introduced beneath the skin in sublethal doses it penetrates the interstices of the connective tissue, at the injection site, to a certain extent. The cells composing this connective tissue, belonging as they do to the first type, do not unite with the toxin in any way and remain passive in respect to it. This is shown by the following points: (*a*) They cannot belong to the second type of cells because there is no detectable necrosis to be observed at the injection site. (*b*) They cannot belong to the third type of cells because the toxin disappears from the connective tissue elsewhere in the body at the same time that it disappears from the blood, whereas it should remain there at least 24 hours after leaving the blood stream, before destruction by the intracellular enzymes. Therefore the presence of toxin in the general connective tissue at the 24- and 48-hour periods is due to mere physical diffusion, which would explain its elimination as soon as the concentration of the toxin in the blood begins to decline. (*c*) It is conceivable that these connective-tissue cells do not enter into combination with the toxin in any way, but secrete an extracellular enzyme capable of breaking up the toxin. That this is not the case, however, is shown by the long persistence of the toxin in the meshes of the connective tissue at the injection site.

A second, and much smaller, portion of the administered toxin unites with the second-type cells of the hair follicles (and perhaps sebaceous and sudorific glands) in that location, and, killing them, produces the alopecia described earlier. This alopecia is slow in appearing because of the low affinity of the a receptors for the toxin, and indeed the only reason it occurs at all with these sublethal doses is because of the large amount of toxin continually held there by the connective tissue meshes. The remainder of the toxin, that is, the excess beyond the holding capacity of the connective tissue, rapidly passes into the blood stream which carries it to all parts of the body. The highly susceptible brain is thus exposed as early as the other tis-

sues, and the reason the animal is not killed is because the brain, in spite of its susceptibility, has but feeble combining powers with the toxin, its cells being of the second type, while other tissues belonging to the third type, such as the liver, etc., which are immune to the intracellular action of the toxin, have a very strong affinity for the latter's binding group and so withdraw all the toxin circulating in the blood, a process which, coupled with excretion by the kidney and liver, and possibly sweat (Salter[15]), leaves none to unite with the weak *a* receptors of the brain. The toxin taken up by tissues containing type-three cells remains intracellular for a period varying with the amount taken in and the activity of the enzymes, until the latter have so changed its chemical composition that it no longer reacts to the biological test. The end products of its katabolism, no longer toxic, are excreted by the cells into the circulation and eliminated by the kidneys, as is the case with other food elements.

To summarize, the immunity of the white rat to large doses of toxin rests upon prevention of the latter from uniting with the highly susceptible nervous tissues because of the greater affinity of other tissues composed of cells insusceptible to the intracellular action of the poison due to a difference in the chemical composition of their protoplasm. A lethal dose of toxin, then, is one which exceeds the capacity of the body's type-three cells to take up the toxin leaving the surplus to circulate in the blood until the slowly acting *a* receptors of the brain become saturated and death ensues—a case quite similar to what happens in the causation of alopecia at the injection site, or upon intracerebral injection of the toxin when the brain tissue is actually bathed in the poison.

If later experiments show that artificial exaltation of immunity is not due to the presence of antitoxins in the blood, it could readily be accounted for by this hypothesis on the basis of a greater capacity of the third type of cells to take up the toxin from the blood, due to a more rapid katabolism of the toxin in these cells because of an increased production of the intracellular enzymes concerned. It is easily recognized that such an increase in the rapidity of enzyme production is borne out by analogy with all that is known of antibody formation.

Finally it may be mentioned that what we know of the part the liver plays in the elimination of poisons in general agrees with the

evidence given in these experiments of its preponderating role in the protection of the rat against this toxin.*

THE RESISTANCE OF THE RABBIT TO DIPHTHERIA TOXIN.

The only data hitherto published upon this point are contributed by Roux and Yersin,[16] who indicate that gram for gram the rabbit is twice as susceptible to the (subcutaneous ?) injection of diphtheria toxin as is the guinea-pig. Finding it necessary to confirm this, a series of rabbits were injected under the skin of the abdomen with varying quantities of toxin, death on the fourth day being taken to indicate the minimum lethal dose. The technic employed was the same as that described above in regard to rats. At autopsy it was observed that rabbits dead of diphtheritic intoxication from subcutaneous injection always show a fibrinous or sero-fibrinous exudate, usually quite abundant in amount, at the site, but no other uniform lesion. The stomach lesion observed by Rosenau and Anderson[13] in guinea-pigs could not be detected in the rabbit. There is frequently, however, a hemorrhagic condition of the adrenals, testes or ovaries and Fallopian tubes.

Examination of Table 5 shows that weight for weight the rabbit and guinea-pig are about equally susceptible to this toxin, and do not

TABLE 5.

RABBITS. SUBCUTANEOUS M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
4.....	1264	.00632	42	2.4	½	+
5.....	1477	.01477	42	2.1	1	5½
19.....	1577	.02712	45	1.5	1	3½
7.....	1250	.025	42	3.0	2	2½
8.....	1205	.03015	42	2.3	3	1½

exhibit the difference stated by Roux and Yersin. The discrepancy perhaps is due to the fact that the latter observers used 400-gram guinea-pigs as standards instead of the present-day test weight. We know now that these larger animals do not exhibit the same uniform-

* It was intended to carry out a similar study of the reactions to diphtheria toxin displayed by a naturally immune poikilotherm—the brown-frog. Experimental difficulties, however, compelled abandonment of the attempt. One or two of the observations nevertheless may be of interest. The brown frog, kept at low temperatures, is not affected by the intraperitoneal administration of 8,000 gram-doses of diphtheria toxin. After 24 hours injected toxin is to be found only in the blood and liver. It will be recalled that Metchnikoff observed that toxin persisted in the blood of green frogs for some two months.

ity in their reaction to toxin as those of 250 grams. Further, their rabbits were considerably larger (3 kg.) than those obtainable here, and, finally, it is quite impossible to determine the minimum lethal dose for rabbits as accurately as for guinea-pigs, partly because of the greater daily fluctuations in weight, due to variations in the quantity of food eaten at the last meal, and in the rate and time of defecation, and partly because of the greater individual variation in resistance in this species. In connection with the close equality in susceptibility to diphtheria toxin shown by the rabbit and guinea-pig, it is interesting to note the extent of the disparity existing between them in regard to tetanus toxin, to which according to Knorr[17] the rabbit is about one thousand times more resistant, gram for gram.

The intraperitoneal (Table 6) and intravenous (Table 7) minimum lethal doses of diphtheria toxin for rabbits were next deter-

TABLE 6.

RABBITS. INTRAPERITONEAL M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
61.....	2155	.0081	46	1.5	$\frac{1}{5}$	..
2.....	1735	.01185	45	1.2	$\frac{2}{5}$	$2\frac{1}{2}$
11.....	1485	.01114	42	2.1	$\frac{3}{4}$	$2\frac{1}{2}$
10.....	1625	.0079	42	3.3	$\frac{1}{3}$	$6\frac{1}{2}$
56.....	2285	.0215	46	1.5	$\frac{1}{2}$	$1\frac{1}{2}$

TABLE 7.

RABBITS. INTRAVENOUS M. L. D. OF DIPHTHERIA TOXIN.

Rabbit No.	Weight	c.c. Toxin Injected	Toxin No.	Diluted To c.c.	No. Gram-doses	No. Days Survived
1.....	1264	0.00632	42	2.4	$\frac{1}{2}$	$1\frac{1}{2}$
3.....	1332	0.02664	42	4.5	2	$\frac{1}{2}$
6.....	965	0.004825	42	1.95	$\frac{1}{4}$	$6\frac{1}{2}$
12.....	1480	0.0049	42	2.5	$\frac{1}{3}$	$4\frac{1}{2}$

mined in a similar manner. Comparison of Tables 5, 6, and 7 shows that the rabbit is about three times as susceptible to this toxin when it is administered intravenously or intraperitoneally as subcutaneously. In other words the tissues at the injection site are capable of holding in some way a quantity of toxin twice as great as the true, or intravenous, M. L. D. The explanation of this phenomenon is by no means the same problem as that encountered in the case of subcuta-

neous injection in the rat, for while the latter belongs to the group of naturally immune mammals, the rabbit is highly susceptible by nature, and therefore each must be studied separately.

If it is the action of the local connective tissue that causes the difference between the subcutaneous and intravenous M. L. D.s, the question is raised why the great amount of connective tissue throughout the body does not protect it against the intravenous M. L. D., since the small amount of connective tissue at the injection site is able to take up in some way twice that quantity of toxin. Certainly toxin introduced into the blood is brought into intimate relation with all the connective tissue of the body, so that the latter might be expected to withdraw all the toxin from the blood and save the animal. That this does not occur indicates that the connective tissue cells are either unable to unite with the toxin at all or else do so very slowly. In either case they would be unable to afford the protection mentioned against subcutaneous injection. To be sure, the necrosis of the connective tissue at the injection site shows that these cells can unite with the toxin, but the fact that there are no focal necroses in the connective tissue after intravenous injection indicates that the selective power of the connective tissue is not great. Physical absorption seems scarcely probable because in that case the rabbit would be able to accommodate more toxin subcutaneously than the white rat, since it has a much larger amount of connective tissue, but, as a matter of fact, the rabbit is vastly more susceptible to toxin given subcutaneously. A more plausible explanation, perhaps, is that the presence of very large quantities of toxin, comparatively, is able to bring about reactions which smaller quantities cannot. Thus the presence of an entire intravenous M. L. D. or double that quantity in the subcutaneous tissue enables the toxin not only to unite with some of the connective tissue cells causing a local necrosis but so to damage the endothelium of the local blood vessels that edema occurs, the coagulation of the fibrinogen therein resulting in the appearance of the fibrinous exudate mentioned above. This exudate may be the real element of defense, and may function either physically, by holding the toxin in its meshes, mechanically, by walling off the toxin-infiltrated area and preventing diffusion of the poison into the body, or chemically, by entering into combination with a certain amount of the

toxin and binding it, thereby shielding the animal; in any case, the result is a capacity to take care of twice as much toxin as the intravenous M. L. D. When more than this is given, either because it diffuses out before the mechanism is in working order or because it is too much for the local powers of accommodation, the excess gets into the blood stream where it circulates in decreasing quantities for about eight hours (de Croly[18]), or perhaps some what longer (Bomstein[19]). While it is thus brought into intimate relation with the connective tissues of the body in general, the circulating toxin is not taken up by them because of the lack of the above two factors to which are due the holding of toxin at the injection-site. In the first place, the requisite degree of toxin concentration is not afforded because of the dilution from mixture with the entire blood supply, and because only a fraction of the toxin administered reaches the blood at all. In the second place, the period of exposure to the toxin is much shorter. Thus de Croly observed that while toxin injected intravenously was present in the blood in practically the entire original amount fifteen minutes later, after two hours only from one-fourth to one-half remained, and it had practically disappeared at the end of eight hours. Bomstein obtained about the same results. After one hour he found in the blood one-half, after three hours one-quarter, and after 12 hours only one-eighth, of the amount injected. This sharp decrease in the toxin content of the blood must be due to the fixation of the toxin by some cells apparently essential to life and possessing a strong affinity for the toxin molecule. According to Bomstein no appreciable quantity of toxin is eliminated by either the urinary or the alimentary systems.

Regarding the possible modes of action of the fibrinous exudate in causing the discrepancy between the subcutaneous and intravenous M. L. D.s, according to the physical-absorption view we would assume that the toxin is taken into the meshes of the fibrin and held there so as to delay but not prevent its reaching the circulation. Practically the full amount of toxin given subcutaneously, therefore, will get into the blood stream, but as it diffuses slowly and since we arbitrarily take death on the fourth day as the criterion, the subcutaneous dose must be so large that the fraction reaching the blood during the first few days equals the intravenous M. L. D. The hypothesis of

mechanical action would consist merely of a variation upon the foregoing; the fibrin of the exudate forms a wall or barrier impervious to the fluid which prevents diffusion of the toxin, after the manner of peritoneal adhesions limiting a localized infection like appendicitis, instead of acting as a sponge as would be the case if the first view were true. A toxic dose would be one large enough to permit the diffusion of an intravenous M. L. D. before the fibrinous exudate had completed walling off the injected area. It is to be noted in this case that the remainder of the toxin would never reach the circulation, as would eventually occur in the case of physical absorption. The hypothesis of chemical neutralization or binding of the toxin by the fibrin needs no explanation except that here also a lethal dose would be one large enough to permit the diffusion of an intravenous M. L. D. before enough fibrin has been formed to accomplish its purpose of neutralization.

Although, as stated above, it appears improbable that this protection is due to mere physical absorption of the toxin by the local connective tissue, after the manner of the protective action of charcoal, for example, it was thought advisable to obtain some experimental evidence upon the point. It seems reasonable that if the local connective tissue is able to absorb twice the intravenous M. L. D. or two-thirds of the subcutaneous dose, the injection at different points of two or three doses whose individual value is slightly less than this figure but whose sum exceeds the subcutaneous M. L. D. ought to have no particularly deleterious effect upon the rabbit, since at each injection-point the local connective tissue is able to hold more than the injected amount. To test this a rabbit was given three subcutaneous injections simultaneously at different points on the ventral surface, each injection equal to one-half the subcutaneous M. L. D. and therefore individually less than the difference between the subcutaneous and intravenous M. L. D. but collectively amounting to 50 per cent more than the subcutaneous M. L. D. The animal succumbed acutely in three days, showing that this hypothesis is inadequate. On the other hand, if the mechanism is either physical absorption or mechanical walling-off by fibrin, the administration of a dose large enough to induce fibrin formation but not so great as seriously to hurt the animal, and followed after four to seven days, when the fibrinous

exudate is completely formed, by a second and lethal dose, should have no effect upon the animal, since its protective mechanism would be in working order. A number of experiments performed in another connection (Table 8) show that neither of these views are tenable. The conclusion then, seems unavoidable that (*a*) the protection afforded by the connective tissue is due to the formation of fibrin, and (*b*) the action of the fibrin is chemical rather than physical or mechanical.

By the hypothesis of chemical action the results of the last-quoted experiments may be easily explained, since the fibrin formed by the stimulus afforded by each injection is largely neutralized by the toxin in the process of uniting with it, and therefore another larger dose would have almost the same effect upon the animal as if no fibrin had been previously formed.

Normal rabbit serum does not protect guinea-pigs against a lethal dose of toxin, so it is evident that the presence of free antitoxin in the blood is not a factor in the mechanism of the rabbit's resistance to diphtheria toxin.

IMMUNIZATION AND HYPERSUSCEPTIBILITY OF RABBITS TO DIPHTHERIA TOXIN.

The literature contains no mention of any previous attempts to obtain an artificial immunity in rabbits to diphtheria toxin, although the subject of the possibility of artificial immunization of naturally susceptible animals to bacterial poisons is of great importance because of its bearing upon vaccination and serum therapy. As for other mammals naturally susceptible to diphtheria toxin, it is familiar that the horse, goat, and cow can be immunized readily while the guinea-pig is somewhat refractory to the process and can be immunized to but a limited extent.

In a series of experiments designed to test the possibility of rabbit immunization (Table 8), in spite of varying the size of the initial dose, the gradual increase in the amount administered at each injection and the time interval between injections, it has thus far been found impossible to diminish the rabbit's susceptibility to the M. L. D. The variations in the initial dosage ranged from $\frac{1}{5}$ to 1/10,000 of the M. L. D. (either subcutaneous, intravenous, or intraperitoneal,

as the case might be), while the interval between injections varied from 1 to 14 days.

TABLE 8.

Rabbit No.	Mode of Injection	Injections in M. L. D.	No. Days Interval	Days Survived after Last Injection	Sum of All Injections in M. L. D.
27	S	$\frac{1}{1000}$, $\frac{1}{500}$, $\frac{1}{250}$, $\frac{1}{125}$, $\frac{1}{60}$, $\frac{1}{30}$, $\frac{1}{15}$, $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, 1.	1	$3\frac{1}{2}$	2
28	S	$\frac{1}{1000}$, $\frac{1}{500}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{1}{2}$, 1, $1\frac{1}{4}$	1	$1\frac{1}{2}$	$3\frac{2}{5}$
33	S	$\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{1}{2}$, 1	1–2	$2\frac{1}{2}$	$2\frac{3}{20}$
32	S	$\frac{1}{10000}$, $\frac{1}{5000}$, $\frac{1}{2500}$, $\frac{1}{1250}$, $\frac{1}{625}$, $\frac{1}{300}$, $\frac{1}{150}$ $\frac{1}{75}$, $\frac{1}{40}$, $\frac{1}{20}$, $\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{3}$, $\frac{2}{3}$	1–7	$4\frac{1}{2}$	$1\frac{2}{5}$
29	S	$\frac{1}{10}$, $\frac{1}{8}$, $\frac{1}{6}$	3–4	$14\frac{1}{2}$	$1\frac{57}{120}$
30	S	$\frac{1}{8}$, $\frac{1}{6}$	3	3	$\frac{2}{5}$
34	S	$\frac{1}{8}$, $\frac{1}{6}$, $\frac{1}{5}$	2–3	$4\frac{1}{2}$	$\frac{3}{5}$
31	S	$\frac{1}{6}$, $\frac{1}{3}$	7	5	$\frac{1}{2}$
35	S	$\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{3}$	8	$4\frac{1}{2}$	$\frac{19}{30}$
37	S	$\frac{1}{20}$, $\frac{1}{10}$, $\frac{3}{20}$, $\frac{1}{5}$, $\frac{1}{4}$	3–4	$3\frac{1}{2}$	$\frac{3}{4}$
36	S	40 injections of $\frac{1}{100}$ each	1	Survived	$\frac{2}{5}$
42	P	$\frac{1}{100}$, $\frac{1}{50}$, $\frac{1}{25}$, $\frac{1}{12}$, $\frac{1}{6}$, $\frac{1}{3}$, $\frac{2}{3}$, 1	1–3	$1\frac{1}{2}$	$2\frac{1}{3}$
41	P	$\frac{1}{10}$, $\frac{1}{8}$, $\frac{1}{6}$	7	$8\frac{1}{2}$	$1\frac{57}{120}$
62	I	$\frac{1}{10}$, $\frac{1}{8}$	8	$2\frac{1}{2}$	$\frac{1}{5}$

S=subcutaneous. P=intraperitoneal. I=intravenous.

Preliminary treatment with large quantities of antitoxin has apparently but little power to confer a lasting passive immunity. This is shown by an experiment in which two rabbits were each given 3,000 units of diphtheria antitoxin in three equal doses on alternate days. After one week the animals were bled and the serum tested for antitoxin. None could be demonstrated. Each rabbit was then given ten rabbit M. L. D.s. of toxin. One died the following day, the other survived, and was given twenty times the M. L. D. a week later. It was found dead the next morning. The experiment shows, however, that the administration of antitoxin, notwithstanding its disappearance from the blood, confers a little immunity.* This method of immunization is of course quite different physiologically from that in which repeated doses of the toxin itself are employed.

Not merely has it been found impossible, as shown above, to produce any artificial immunity by repeated injection of toxin, but the rabbits were found to develop, in some cases, a supersensitiveness or hypersusceptibility to the toxin which caused their death upon administration of sublethal doses (cf. Table 8). This occurred with intraperitoneal and intravenous as well as subcutaneous injections.

* Experiments are planned to determine accurately how high this degree of immunity is, what ratio t bears to the amount of antitoxin administered, and how long antitoxin can be detected in the serum.

That this phenomenon is a true hypersusceptibility and not a mere cumulative action, such as discussed in connection with the white rat, is shown by the fact that a number of these rabbits died before the sum of all the doses administered had reached the lethal quantity. Inasmuch, therefore, as no cumulative action was concerned in these cases, there is justification for the assumption that it was absent in the other cases as well, even where the sum of all the doses administered exceeded the lethal quantity, and that the rabbit was killed by the last dose given rather than by a summation of all the quantities injected.

Hypersusceptibility to toxin injections has been observed in other animals to a much more marked degree than occurs in the case of the rabbit to diphtheria toxin, as shown by experiments described above. Behring and Kitashima[20] report that guinea-pigs, started with the injection of 1/400,000 M. L. D. of diphtheria toxin, die upon receiving from 1/800 to 1/700 M. L. D., although the sum of all the injections amounted to but 1/400 M. L. D. This result is of course far more pronounced than that of the above experiment on rabbits. The authors show further that evidences of hypersusceptibility to this toxin can be obtained in horses and apes, and also cite the case of a horse that died of diphtheria intoxication during the process of immunization, although the blood possessed antitoxin. Rist[21] injected small quantities of dried diphtheria bacilli into guinea-pigs and found that the first dose produced but a transient illness, the second a more protracted one, and the third caused death. Brieger[22] reports the case of a goat that died of tetanus toxin although immunized to such an extent that considerable quantities of antitoxin were found in the blood and milk. Knorr[23] was able to produce so great a hypersusceptibility in guinea-pigs toward tetanus toxin that even 1/50 M. L. D. might cause death. It is interesting to note that his experiments on rabbits not merely produced no hypersusceptibility but immunized them to the extent of withstanding five times the M. L. D. of tetanus toxin. Finally there is the so-called "paradoxical phenomenon of Kretz," quoted by Wassermann,[24] that while normal animals do not react to a mixture of toxin and antitoxin in certain proportions, animals previously immunized to that toxin will react to the mixture, due, no doubt, to incomplete neutralization of the toxin by the antitoxin, leaving but a sublethal quantity of toxin free which is too small to affect normal animals but will injure those made hypersusceptible.

In regard to the results shown in Table 8, the first thing to be done is to determine which factor in the procedure causes the phenomenon. To see if the effect of the mere number of injections could be responsible, a rabbit was given, for over a month, daily injections of 1.5 c.c. of sterile physiological salt solution, as it was to this volume that all toxin injections in the hypersusceptibility series were diluted with salt solution. The animal not merely showed no inconvenience from the procedure, but gained in weight quite notice-

ably, so it is evident that the mere effect of the repeated injections cannot account for any part of the findings. The next element to be considered was the broth conveying the toxin. As this may have caused an anaphylaxis, similar to that exhibited toward sera, corpuscle extractives, etc., a rabbit was given twelve injections of 0.5 c.c. of Theobald Smith's[25] diphtheria infusion broth (which was used for the production of the toxin) plus 1 c.c. of salt solution. The animal thrived and gained weight during the experiment. As the hypersusceptibility to foreign albumins is often most marked following the repeated injection of very small quantities, another animal was submitted to the same procedure, except that only 0.1 c.c. of the infusion broth and 1.4 c.c. of salt solution were given. This animal also gained weight. Since, then, neither the broth nor the effect of the repeated injections is able to lower the vitality of the animal, the only alternative is that the hypersusceptibility is to the toxin alone.

Several theories have been advanced to explain the phenomenon of hypersusceptibility, when it occurs following injections of foreign sera (anaphylaxis, serum disease). Thus Bail[26] holds that repeated injections of a serum causes the production of a substance which prevents the leucocytes from fulfilling their normal rôle of protection. Richet[27] believes that serum contains two bodies, one of which tends toward the production of immunity, and the other of the phenomenon of hypersusceptibility in the animal injected. Courmont[28] advances the theory that the first injection causes some protective substance to be absorbed, so that when the second injection is made there is nothing to neutralize the toxic principle in the serum. This theory is elaborated by Currie,[29] who holds that the active principle in the injected serum gives rise to an antibody, or rather causes one to be produced by the animal, union with which forms the true toxic principle. When but one injection of serum is made, the antibody is produced so gradually that the body is either able to eliminate the combined form or else to produce another antibody to neutralize the toxic compound resulting from the serum substance and the first antibody. If a second injection of serum is made, however, enough of the first antibody is left over from the effects of the first injection to produce intoxication, either because there is too much to be eliminated quickly or because the second antibody has disappeared from the body

during the interval. Wright,[30] observing a fall and then a rise in the bactericidal power of the blood after inoculation with vaccine, holds that hypersusceptibility may be explained by the injection of a sufficient quantity of the immunizing agent during the negative phase to produce a cumulation.

As shown above, however, cumulation cannot be the explanation of hypersusceptibility to toxin, and the experiments of Behring and Kitashima[20] on guinea-pigs corroborate this. That it does not apply even to serum hypersusceptibility is seen from the experiments of Otto[31] and of Rosenau and Anderson,[32] so that Wright's hypothesis cannot be accepted. Since Otto and Dungern[33] have shown that precipitins do not appear in the blood at the time of hypersusceptibility, this explanation also must be discarded, and, as pointed out by Currie,[29] the theories of Courmont, Richet, and Bail fail to take account of the time interval between injections, a factor apparently quite significant, as Table 8 indicates that little or no hypersusceptibility was shown by the animals injected at short intervals. While data upon this point are as yet incomplete, a comparison of Rabbits 29 and 41 in Table 8 shows that an interval of three or four days is just as effective in producing a hypersusceptibility as a longer period, while the records of Rabbits 30 and 34 show that two days is not as effective as three. These assumptions appear to be borne out by the other animals, though not in a manner so easy to interpret.

Although Currie's theory may account for the phenomenon when it is occasioned by repeated injections of serum, it is apparent that it cannot hold for the phenomenon elicited by diphtheria toxin. The mere fact that a single dose of toxin, if sufficiently large, is capable of killing a rabbit in a few hours shows that it is not dependent for its toxicity upon union with a slow-forming antibody, which is the basis of Currie's hypothesis.

The explanation of the phenomenon, as regards supersensitization to toxin, appears to me to be much simpler. If we look upon the body cells as belonging to three types, nervous tissue cells, essential to life, which are able to bind the toxin, cells not essential to life which are also able to bind the toxin but have a higher affinity for the toxin than the nervous tissue, and finally neutral cells which are inert in respect to the toxin, the question will be easier of solution.

Because of greater affinity the vitally-unessential cells of the second class will take up all the toxin introduced into the animal as far as their saturation limit, and of a toxin dose within this limit none is able to reach the vital nervous tissue. A lethal dose of toxin is one which so exceeds this limit that enough toxin is left over to unite with enough nervous tissue to cause death. If we let 1 equal the quantity of toxin sufficing to destroy enough of the nervous tissue, when allowed to reach it, to kill the animal, and let 9 equal the amount of toxin which the less essential, but chemically more active, cells can take up, then the injection of 9 units of toxin will injure the latter cells, but since they are not necessary to life the animal will not suffer seriously. A lethal dose would be $9+1=10$ units, as this would leave 1 unit able to reach the central nervous system. If a 9-units dose be given, the protective cells are destroyed, or at least so injured (perhaps by loss of the proper receptors) that the nervous tissue is left without any safeguard and the administration of a single unit would kill the animal. This condition lasts until the protective cells or their damaged receptors are regenerated, a process which, as the foregoing data indicates, takes place very slowly. Hypersusceptibility, or death from doses amounting to less than ten units, can be explained by analogy with well-known pathological processes. Suppose an initial dose of one unit be given a normal animal, one-ninth of the protective tissue receptors are immediately bound and destroyed, and their degeneration may cause the degeneration of another two-ninths of these receptors in the same manner as necrosis of a certain number of cells in an organ is often followed by spreading necrosis of a large additional area, or else it may be caused by interference with nutrition, etc., after the manner in which occlusion of a blood vessel supplying a region produces an anemic infarct. A period sufficient for the development of this degeneration but insufficient for regeneration being allowed to intervene, a second dose of toxin amounting to two of the hypothetical units can, by uniting with two-ninths of the remaining protective tissue receptors, cause the degeneration and sloughing-off of, say, another three-ninths of these receptors, leaving but one-ninth of the protective receptors left to guard the vital central nervous system. After a like interval a third dose of two units is given, one-half of which is bound by the remaining one-ninth of the protective tissue

receptors, while the other half is free to unite with the receptors of the nervous system and thus kill the animal, although only one-half as much toxin has been given in all the doses as is required to kill the animal at a single administration. If objection be offered to the "receptor" terminology employed above, the same hypothesis will hold by assuming an excretory function for the protective cells. It is known that small quantities of certain drugs are able to produce a slow-forming inflammation and degeneration of the organ which excretes them, as the kidney. Thus a large dose is eliminated after injection, but after a while so much harm has been done by the resulting inflammation that a second small dose cannot be eliminated and produces toxic effects. Inasmuch as a smaller dose can produce the same amount of inflammation as the larger, the same amount of poisoning would be produced by a smaller original dose. Applying this to the effect of toxin on rabbits, we might assume that the protective cells are normally able to eliminate in some way, perhaps after a change in chemical character, certain amounts of toxin. The process, however, is so injurious to them that a degenerative change soon develops in the part of the cell carrying out this function which renders it incapable of repeating the process for a considerable time. Then when a fraction of the lethal dose of toxin is given it is eliminated or changed by these cells so that it cannot reach the central nervous system, but in the course of three or four days there appears a degeneration of the eliminating mechanism in these cells lasting some weeks at least, so that when another small fraction is given it cannot be eliminated and thus gets to the nervous tissue, which we know to be susceptible to very small amounts of toxin, and kills the animal. When a single large dose of toxin, sufficient to kill the animal, is given, the mechanism of action is different because the eliminating apparatus is in working order. Death results, however, because the amount of toxin administered is too large to be gotten rid of with sufficient rapidity to prevent the small moiety sufficient for that purpose from uniting with the nervous system.

It seems to me that this theory avoids the objections raised to the other hypotheses cited, in that it explains both the significance of the time interval necessary between injections, and the mechanism of death from the administration of a single large dose.

SUMMARY.

The foregoing is a study of the reactions of a naturally immune mammal, the white rat, and one naturally susceptible, the rabbit, toward diphtheria toxin.

Experiments have shown that the rat is not absolutely immune but that it is killed in four days by 3,500 times the guinea-pig lethal dose, corrected to weight, when injected subcutaneously.

The resistance of half-grown rats is half the above figure, even when corrected for weight.

The intraperitoneal M. L. D. for the rat is 1,200 times the guinea-pig subcutaneous M. L. D.

A local semi-specific alopecia occurs at the site of subcutaneous injection.

I have been able to exalt the natural resistance of the rat by repeated injections of increasing doses of toxin.

No cumulative action or hypersusceptibility is exhibited.

The immunity of the rat is not due to free antitoxins in the blood.

The fate of toxin injected into the rat has been traced in detail, and a theory advanced to explain the mechanism of the rat's immunity.

Subcutaneous, intraperitoneal, and intravenous minimum lethal doses for the rabbit have been ascertained, using the guinea-pig as a standard, and an hypothesis formulated to explain the resistance mechanism.

It is found that rabbits exhibit hypersusceptibility to injections of toxin, whether subcutaneous, intraperitoneal, or intravenous. A theory is advanced to explain this phenomenon.

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ANTIBODIES TO GLUCOSIDES, WITH ESPECIAL REFERENCE TO *RHUS TOXICODENDRON*.*

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SOME of the observations which have been made upon poisonous fungi in the laboratory during the past three years have considerable bearing upon general problems in immunity since the conclusions drawn are in direct contradiction to accepted theories upon this subject. It has been shown,[1] for instance, that the aqueous extract of *Amanita phalloides* contains a powerful hemolysin acting upon a variety of blood corpuscles, and in addition a heat-resistant non-hemolytic toxic substance killing animals acutely and producing fatty degenerations. With this aqueous extract it is possible to immunize animals with the production of a serum which has marked anti-hemolytic and low but nevertheless definite antitoxic properties. The antihemolysin is present in a dilution of 1–1,000 of the serum, and 1 c.c. of this serum will neutralize five to eight times a fatal dose of the extract for rabbits or guinea-pigs. It has since been shown in association with Dr. Abel,[2] that the hemolysin is a *glucoside* extremely sensitive to the action of heat, acids, and the digestive ferments, and

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[1] Ford, *Jour. Infect. Dis.*, 1906, 3, p. 191.

[2] *Jour. Biol. Chem.*, 1907, 2, p. 1.

not a "*toxalbumin*" as supposed by Kobert.[1] All proteid may be removed from this glucoside by metaphosphoric acid and by uranyl acetate, leaving its hemolytic activity unaltered. Now if it be possible to immunize animals to this hemolytic substance, producing an antihemolysin, and if, at the same time, it can be shown that this hemolysin is a glucoside, we have consequently produced an *antibody to a glucoside* the possibility of which has been denied by Ehrlich,[2] Bashford,[3] and a number of others. The contentions of the above-mentioned observers rest upon a large series of attempts to immunize animals to the glucosides saponin and solanin, and to certain of the poisonous glucosides found in digitalis and in ergot. It had been claimed by one or two observers, notably by Pohl,[4] that after the introduction of small doses of solanin subcutaneously in rabbits, the serum of these animals shows an anti-hemolytic action more marked than does the normal rabbit's serum; and it is still believed by Metchnikoff[5] that the serum of animals treated by non-proteid substances like morphine and arsenic has an increased capacity of neutralizing or oxidizing these substances. In general, however, it may be said that antitoxins have thus far been produced only for bodies supposed to be proteid or proteid derivatives.

These observations upon *Amanita phalloides* thus reopen the entire question, since they must either rest upon fundamental errors in laboratory investigation, or the thesis that antibodies cannot be produced for glucosides does not have a universal application. During the past few months, therefore, we have been studying the action of several of these toxic compounds upon animals, and with one of them the results have been sufficiently constant to merit publication. The substance to which especial attention has been paid is the poisonous glucoside found in *Rhus toxicodendron*, or poison ivy. The active principle of this plant was first properly studied by Pfaff,[6] who found that the irritating poisonous properties reside in the alcoholic extract of the leaves and stem, and the poisonous body was thought by Pfaff

[1] *St. Petersburger med. Wchnschr.*, 1891, 16, pp. 463, 471.

[2] *Chemical Constitution and Pharmacological Action: Collected Studies on Immunity*, New York, 1906, p. 433.

[3] *Arch. internat. de. Pharm. et de Ther.*, 1901, 8, p. 101; 9, p. 451.

[4] *Arch. internat. de Pharm. et de Ther.*, 1900, 7, p. 1; 1901, 8, p. 437.

[5] *Immunity*, Cambridge, 1900.

[6] *Jour. Exper. Med.*, 1897, 2, p. 181.

to be of the nature of a non-volatile oil. It has recently been shown by Syme,[1] working under Acree in Remsen's laboratory, that the active principle of *Rhus toxicodendron* is in reality a *glucoside*, a compound of rhamnose, gallic acid, and fisetin. It is precipitable by lead acetate and upon hydrolysis yields the above substances. The toxic action of the various fractions was tested by Syme upon his own skin, and in this way he was able to follow the poison through the various chemical procedures necessary for its isolation.

Certain clinical phenomena are of interest in connection with poisoning by *Rhus toxicodendron*, and there are vague suggestions in the experience of a number of individuals pointing to an acquisition of artificial immunity. With many persons a small amount of the juice from the fresh leaves is capable of setting up an extensive inflammation of the skin, while others seem able to handle the plant with impunity. At the same time individuals who have been severely poisoned by poison ivy, after a certain number of attacks become accustomed to it, to such an extent at least as no longer to suffer the same serious consequences as at first. Again, certain observers claim to have been cured of an attack and to have been rendered insusceptible to further attacks by the administration of the fluid extract of *Rhus toxicodendron* internally, or by chewing the fresh leaves of the plant. Finally Syme, in his experiments upon himself, found that after four to five months he was no longer susceptible to the poison, although originally his skin had been extremely sensitive. All of these observations are valuable, and while by no means conclusive in showing that immunity may be acquired to this poison, they certainly point out a line for further investigation.

I was enabled to obtain the active principle of *Rhus toxicodendron* in the alcoholic fluid extract of the native plant as prepared by Parke, Davis & Co. This fluid extract contains the poison in workable and practically constant proportions. It had already been shown by Pfaff that the internal administration of his non-volatile oil produced definite lesions in rabbits, the animals dying of an acute nephritis at the end of 14 to 15 days. Occasionally the rabbits died in acute convulsions without any macroscopic brain lesions. The subcutaneous administration of the fluid extract of *Rhus toxicodendron*

[1] *Some Constituents of the Poison Ivy Plant (Rhus intoxicodendron)*, Johns Hopkins Thesis, 1906.

produces the same effects upon rabbits as those described by Pfaff. Rarely, the rabbits die in convulsions within 24 to 48 hours, but the majority of inoculated animals succumb in from 8 to 15 days. In addition to the nephritis an extensive necrosis and slough is found at the point where the poison is introduced beneath the skin. Following the inoculation we have a fairly long latent period during which the weight of the animals remains stationary. After seven or eight days in a typical case, the animal loses weight rapidly, the necrosis and slough develop, and the animal dies of the nephritis after the lapse of about two weeks. At times the skin lesions are less marked, the damage to the kidney being the important change; an intraperitoneal inoculation seems able to produce these kidney changes with more rapidity than does the subcutaneous method. In addition to rabbits we have found that guinea-pigs are susceptible to the drug, the lesions being produced with greater certainty and regularity. With these animals the necrosis and slough at the point of inoculation are more extensive, while the animals die of the kidney changes in about the same time. The fatal dose of the poison can be estimated for both animals with tolerable accuracy. For guinea-pigs of 250 gram weight, 0.25 c.c. of the alcoholic extract always represent a fatal dose; and a guinea-pig of 350 gram weight practically never survives a dosage of 0.5 c.c. With larger guinea-pigs the proportion of poison to body weight is retained (Table 1).

TABLE 1.

TOXICITY OF *Rhus Toxicodendron* (ALCOHOLIC EXTRACT).

Guinea-Pigs.

Grams Weight	Dosage	Result
	c.c.	
250	1	Death, 13 days
370	0.5	Death, 7 days
265	0.25	Death, 3 days
320	0.20	Recovery
290	0.1	Recovery
380	1	Death, 13 days
350	0.5	Death, 9 days
325	0.25	Death, 10 days

The fatal dose for guinea-pigs can be estimated with considerable accuracy. It may be considered 0.25 c.c. for animals of 250 gram weight; 0.5 c.c. for animals of 350 gram weight.

The fatal dose for young rabbits weighing less than 800 grams can

also be estimated fairly accurately. The animals may die from 0.25 or from 0.5 c.c., but since it is rarely the case that rabbits of this weight may survive this quantity our constantly fatal dose must be placed somewhat higher—at 1 c.c. for a rabbit of 800 grams (Table 2).

TABLE 2.

TOXICITY OF *Rhus Toxicodendron* (ALCOHOLIC EXTRACT). Rabbits.

Grams Weight	Dosage	Result
	c.c.	
800.....	1	Death, convulsions, 24 hrs.
800.....	1	Death, 10 days
1200.....	1	Death, 9 days
920.....	0.5	Death, 5 days
855.....	0.25	Death, 5 days
850.....	0.25	Recovery
1550.....	1.5	Recovery
1120.....	0.2	Recovery
920.....	0.1	Recovery
1085.....	1	Recovery
1070.....	0.5	Recovery
1330.....	0.75	Recovery

The fatal dose for rabbits of 800 grams is 1 c.c. Certain rabbits of this weight die from smaller doses, but not regularly, and larger animals show greater resistance. Animals of 1,800 to 2,000 gram weight occasionally survive 2 to 3 c.c. doses, but not more than this amount.

Having established the limits of the fatal dosage for both guinea-pigs and rabbits, one or two preliminary experiments were made to determine whether animals which had withstood small doses of the poison were susceptible to amounts of the poison capable of killing untreated animals. For this purpose two small guinea-pigs weighing about 250 grams, which had received 0.2 c.c. and 0.1 c.c. of the *Rhus toxicodendron* extract, were subsequently given 0.5 c.c. and 0.75 c.c. of the poison. One of these animals died in about eight days after the second dose of 0.5 c.c., no immunity being established. The other animal which had received the lower initial dose of 0.1 c.c. reacted well to the second dose of 0.5 c.c. and again to a third dose of 0.75 c.c. representing two to three times a fatal dose. Its condition remained perfectly good, no necrosis or slough appeared at the site of any of the inoculations, and after a period of two weeks the weight which had remained stationary gradually increased to a point above the original weight and remained at this high point during the time

of observation (about three months). At the same time a rabbit weighing 1,550 grams, which after an inoculation of 1.5 c.c. had developed a huge slough at the site of inoculation and a loss in weight from 1,550 to 1,125 grams, but which had later recovered, was given, seven weeks after its original dose, another inoculation of 2 c.c. The second inoculation, larger than the first, with the body weight considerably diminished, produced no slough at the point of injection, and for three weeks the animal remained in good condition. It died then of an infection, and at the autopsy there was no necrosis at the site of inoculation and no evidence of nephritis.

These two animals were suggestive, therefore, in indicating the acquisition of resistance after non-fatal doses, and in giving us certain data for the more serious attempt to establish a high degree of immunity.

The experiments upon this problem were undertaken with four guinea-pigs varying in weight from 450 to 900 grams, and upon nine rabbits varying in weight from 800 to 2,800 grams. In some cases rabbits which had recovered from large initial doses, in testing the limits of the fatal dose, were deliberately given large amounts to test their resistance, while in other instances fresh animals in good condition were employed and the immunization conducted in a more conservative manner. Of these nine rabbits one large Belgian hare died of infection after a final administration of 6 c.c. of the fluid extract. Two rabbits died during the treatment, both after a dose of 6 c.c. With six rabbits in which the amount of the poison given was gradually increased a definite immunity was established.

In these cases the initial doses were small and frequently repeated, and at first a slight loss in weight was noticeable. When the original body weight was regained the immunization was pushed and larger doses were given. The large doses were not given except at fairly infrequent intervals and only after the loss in weight had been made up. With these precautions we were able gradually to give very large amounts of the *Rhus toxicodendron*, amounts so large in all cases as to do away with the factor of high natural resistance on the part of any of the animals.

Thus two of the rabbits had an initial weight of less than 1,000 grams (920 and 850 grams), and in these animals we were able eventu-

ally to give 6 and 7 c.c., representing at least five or six times a fatal dose for rabbits of their weight (Table 3). No subcutaneous lesions developed after even these large doses; the weight of the animals gradually but surely increased, and after they were killed for the collection of their blood serum, there was no evidence of nephritis, the urine being quite free from albumen. These animals received altogether no less than 30 c.c. of the *Rhus toxicodendron* extract, over a period of four months.

TABLE 3.

IMMUNIZATION OF RABBIT (RABBIT 5, WEIGHT 850 GRAMS).

Date	Dosage (*Rhus tox.*)	Grams Weight
	c.c.	
November 5	0.25	850
December 10	0.50	800
" 12		990
" 19	1.0	935
" 21		1000
" 26	1.3	990
" 27		1030
January 4		1095
" 7	1.6	
" 8	No lesion site of inoculation	1140
" 16	2.0	1220
" 25	2.6	1280
February 2	3.25	1300
" 11	4.00	1320
" 21	5.50	1340
" 22		1420
" 25		1400
March 6	7.00	1380
" 16	Killed	1370

An animal with an initial weight of 1,120 grams was eventually given a dose of 8.5 c.c. and killed 10 days after the last inoculation (Table 4). In this case there was a slight amount of albumen in the

TABLE 4.

IMMUNIZATION OF RABBIT (RABBIT 4, WEIGHT 1,120 GRAMS).

Date	Dosage (*Rhus tox.*)	Grams Weight
	c.c.	
November 15	0.2	1,120
December 11	0.8	1,425
" 19	1.2	1,545
" 26	1.6	1,500
" 27		1,635
January 4		1,600
" 7	2.0	
" 8	No lesion at site of inoculation	1,580
" 9		1,635
" 16	2.50	1,695
" 25	3.00	1,835
February 2	4.0	1,830
" 11	5.50	1,780
" 22	7.50	1,790
March 6	8.50	1,930
	Killed March 16; weight 1,890	

urine, but no necrosis at the site of inoculation. Finally three other rabbits weighing originally 1,250, 1,550, and 2,700 grams were pushed to the point where 10 c.c. of *Rhus toxicodendron* was given (Table 5).

TABLE 5.

IMMUNIZATION OF RABBIT (BELGIAN HARE, NO 2; WEIGHT 2,700 GRAMS).

Date	Dosage (*Rhus tox.*)	Grams Weight
	c.c.	
December 4	0.1	2,700
" 6	0.2	
" 10	0.4	2,500
" 14	0.7	2,930
" 15		2,750
" 17		2,920
" 19	1.0	
" 27		2,785
January 4		2,725
" 5	1.5	
" 7		2,850
" 16	2.0	3,010
" 25	3.0	3,060
February 2	4.50	3,000
" 11	6.00	3,060
" 22	8.00	2,950
March 6	10.00	3,010
" 16	Bled 20 c.c.; Killed March 19	2,950

This being such a large and definite multiple of a fatal dose, allowing for any possible hyper-resistance, the immunization was discontinued at this point and the animals killed about two weeks after the last inoculation. These excessively large amounts of *Rhus toxicodendron* were given in divided doses in different parts of the body.

In these three cases the total amount of the poison given was nearly 40 c.c. of the fluid extract. At the same time the four guinea-pigs were also treated by gradually increasing doses up to the point where they had received 2.5 and 3 c.c. of the poison (Table 6). In one

TABLE 6.

IMMUNIZATION OF FOUR GUINEA-PIGS.

(Weight of guinea-pigs varies from 460 to 950 grams.)

Date	Dosage (*Rhus tox.*)
	c.c.
January 16	0.1
" 19	0.2
" 23	0.3
" 28	0.5
February 4	0.75
" 11	1.00
" 21	1.35
March 2	1.80
" 11	2.50
" 19	Two smaller animals killed
" 20	3.00 given to other animals Killed April 3

of these a slight area of necrosis developed in the skin, while in the others the skin remained normal. After the animals were killed, no albumen or but a trace was found in the urine. From these experiments it may be safely concluded that in both rabbits and guinea-pigs an active immunity to *Rhus toxicodendron* may be established, the final amount of the poison administered being at least six or seven times a fatal dose. Beyond this point there is little object in pushing the immunization since such large volumes of the poisonous extract would be necessary as to damage the skin and subcutaneous tissues before neutralization. Furthermore we have given a sufficiently high multiple of a fatal dose as to indicate a definite artificial active immunity. We have therefore in *Rhus toxicodendron* a poisonous glucoside exerting a selective action upon the epithelial cells of the skin and kidney, by the gradual introduction of which immunity to fatal doses may be established.

NATURE OF *RHUS TOXICODENDRON* IMMUNITY.

Since we are working with a non-proteid substance it is necessary to inquire particularly into the nature of the immunity here described.

Are we dealing with an increased resistance on the part of these animals similar to that shown to morphine, arsenic, and to certain alkaloids like cocaine, or with a real immunity in which the body has produced substances actually uniting with and neutralizing the poison? This is an important question, since it has always been supposed that animals may become accustomed to a number of non-proteid poisons without producing any antitoxin for them. It was necessary therefore to test the blood serum of these immune animals, and to determine what capacity it had, if any, of neutralizing fatal doses of the poison for susceptible animals. For this study guinea-pigs were used exclusively. The great variation in susceptibility of rabbits, which I have already pointed out, would render experiments with them exceedingly difficult to interpret. Fortunately with guinea-pigs we can estimate the fatal dose within fairly narrow limits. Nevertheless we are confronted with a difficult problem, since it is impossible to mix the serum and the poison in varying proportions, as is done with ordinary antitoxins. It must be remembered that we have an alcoholic fluid extract of *Rhus toxicodendron* upon the addition of serum

to which a turbid precipitate is at once produced. It was determined to give the poison and the serum separately in different parts of the body, and I have preferred to employ the severer test of giving the poison first and the serum subsequently. The immune rabbits were bled at appropriate intervals, preferably 12 to 14 days after an injection, and the serum thus obtained was employed upon guinea-pigs to determine its neutralizing powers. In all cases we have found that the serum from these immune animals is antitoxic, and will neutralize definite multiples of fatal doses. Thus in the serum marked Rabbit No. 4, Table 7, it will be seen that 1 c.c. of this serum neutralizes 0.5 c.c., 0.75 c.c., 1 c.c. and 1.5 c.c. of the poison, while 2 c.c. of the

TABLE 7.

ANTITOXIC PROPERTIES OF SERUM FROM IMMUNE ANIMAL.

Serum from Rabbit 4, Bled after Dosage of 4.50 c.c. Tested on Guinea-Pigs.

Grams Weight	Dosage	Result
260	1 c.c. serum+0.5 c.c. *R. tox.*	No effect
305	1 c.c. serum+0.75 c.c. *R. tox.*	No effect
405	1 c.c. serum+1 c.c. *R. tox.*	No effect
405	1 c.c. serum+1.5 c.c. *R. tox.*	No effect
515	2 c.c. serum+2 c.c. *R. tox.*	No effect

CONTROL.

Guinea-pig given 1 c.c. *R. tox.* found dead on 13th day.

The *Rhus toxicodendron* is given subcutaneously and the serum after the *Rhus toxicodendron*, but on the other side of the body.

serum will neutralize 2 c.c. of the *Rhus toxicodendron*. Therefore 1 c.c. of the serum will neutralize five or six times a fatal dose for the animal in question. These test animals were kept under observation for a period of six weeks. With the serum from another immune animal larger quantities of the *Rhus toxicodendron* were employed. As may be seen from Table 8, Rabbit No. 9, 2 c.c. of the serum neutralized 3 c.c. of the *Rhus toxicodendron*, but some necrosis developed at the point of inoculation. One c.c. of the serum neutralized 2.5 c.c. of the *Rhus toxicodendron*, however, with no slough at the site of injection, while with smaller quantities of the serum the neutralization was not accomplished. The same animal, Rabbit No. 9, was further immunized, a final dose of 10 c.c. being given. After this large dose its serum had an increased power of neutralizing the poison. With 1 c.c. and 0.5 c.c. quantities 2.5 c.c. of the poison was

TABLE 8.

ANTITOXIC PROPERTIES OF SERUM OF RABBIT No. 9; BLED 11 DAYS AFTER A DOSAGE OF 7.50 c.c. *Rhus tox.*

(Tested Feb. 25 and March 2.)

Grams	Dosage	Result
600.....	2 c.c. serum+3 .c.c. *R. tox.*	Some necrosis at site of inoculation. Complete recovery
530.....	1 c.c. serum+2.5 c.c. *R. tox.*	No effect
400.....	0.5 c.c. serum+2.5 c.c. *R. tox.*	Death, 16 days
400.....	1 c.c. serum+1 c.c. *R. tox.*	No effect
420.....	1 c.c. serum+0.5 c.c. *R. tox.*	Death, but no lesions of *Rhus tox.* No slough and no nephritis
	CONTROLS.	
530.....	2 c.c. *Rhus tox.*	Death, 17 days
410.....	0.5 c.c. *Rhus tox.*	Death, 4 days
380.....	0.25 c.c. *Rhus tox.*	Recovery

neutralized to such an extent as to show no slough where the poison was injected, the animals dying, however, at a late date from nephritis. One c.c. again completely neutralized 1.5 c.c. of the poisonous extract and 0.25 c.c., a dose of 2 c.c.

It is thus seen that the serum of these animals immunized to *Rhus toxicodendron* contains substances neutralizing this poisonous glucoside when both are injected into susceptible animals, and the immunity is therefore an antitoxic immunity.

Sera from a number of other animals immunized to this poison have been tested and in all cases they have been found to be antitoxic in character. The tables already given are typical of the reactions of such sera and probably represent as strong a serum as small animals can be made to produce. Larger animals, such as goats, have already been immunized and the antitoxic strength of their serum will be tested as soon as opportunity is afforded. Should serum from large animals have a high degree of antitoxic power, the use of such serum would be justified in severe cases of poisoning by *Rhus toxicodendron*, and there is a reasonable possibility that it may acquire a definite place in practical therapy.

THE SPECIFIC NATURE OF ANAPHYLAXIS.*

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From our first studies upon hypersusceptibility we were interested in the question "Is this reaction specific?"

In our work upon the toxic action of horse serum[1] we showed that this reaction is quantitatively specific for serums. That is, guinea-pigs sensitized with horse serum are subsequently very susceptible to a second injection of horse serum, but only slightly if at all susceptible to a second injection of the serum of other animals, such as rabbit, cat, dog, hog, sheep, chicken, or man. Conversely, guinea-pigs sensitized with the serum of these other animals are not very sensitive to a second injection of horse serum, whereas, they respond actively to a subsequent injection of the same serum used for the first injection.

We have further shown[2] that the specific nature of this phenomenon is more marked when proteid substances of widely different nature are used at the first and second injections. Thus a guinea-pig sensitized with horse serum does not react to a subsequent injection of egg-white, vegetable proteid, or milk. A guinea-pig sensitized with egg-white does not react to a subsequent injection of horse serum, vegetable proteid, or milk. A guinea-pig sensitized with milk does not react to the other proteid substances mentioned.

We have recently succeeded in demonstrating more clearly the specific character of the phenomenon we are studying by proving that guinea-pigs may be in a condition of anaphylaxis to three proteid substances at the same time. For instance, a guinea-pig may be sensitized with egg-white, milk, and horse serum and subsequently react to a second injection of each one of these substances. The guinea-pig may be sensitized by giving these strange proteids either at the same time or at different times, in the same place or in dif-

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[1] *Hyg. Lab., Bull. 29*, U. S. Pub. Health & Mar.-Hosp. Serv. Washington, April, 1906, pp. 1–95.

[2] *Hyg. Lab., Bull. 36*, U. S. Pub. Health & Mar.-Hosp. Serv. Washington, April, 1907, pp. 1–67.

ferent places, or by injecting them separately or mixed. The guinea-pig differentiates each anaphylactic-producing proteid in a perfectly distinct and separate manner. The animal is susceptible to the second injection of each one of the three substances in the same sense that it is susceptible to three separate infectious diseases.

These experiments, which we now wish to record, were divided into three series. In the first, the guinea-pigs were sensitized with horse serum, egg-white, and milk, in different parts of the body but at the same time. In the second series the three substances were thoroughly mixed and then injected into the guinea-pig. In the third series the animal was first given an injection subcutaneously of milk, 14 days later an injection of egg-white, and 14 days after that an injection of horse serum.

All of the animals, after at least 14 days from the last injection, were then given the second injection of one of these substances. Those animals that survived the injection of the first substance were

TABLE 1.

GUINEA-PIGS SENSITIZED AT THE SAME TIME, BUT IN DIFFERENT PLACES.

May 15, 1907, the following guinea-pigs received 1/250 c.c. of normal horse serum, 1 c.c. of a solution of egg-white in normal salt solution, and 1 c.c. of whole milk; the injections were given subcutaneously at about the same time but in different parts of the body. The animals were subsequently tested as follows:

Date	No. of G.-P.	Amount	Substance Injected	Method of Inoculation	Interval in Days since First Injection	Result
June 10, 1907	1,008	6 c.c.	Horse (roan) serum	Peritoneum	26	Very severe symptoms
" 11, 1907	1,008	6	Whole milk	Subcutaneous	27	Severe symptoms
" 12, 1907	1,008	6	Egg-white in salt solution	Peritoneum	28	Dead 25 minutes
" 11, 1907	1,009	6	Whole milk	Subcutaneous	27	Marked symptoms
" 12, 1907	1,009	6	Egg-white in salt solution	Subcutaneous	28	Severe symptoms
" 13, 1907	1,009	6	Horse (roan) serum	Peritoneum	29	Marked symptoms
" 11, 1907	1,010	6	Egg-white in salt solution	Subcutaneous	27	Severe symptoms
" 12, 1907	1,010	6	Whole milk	Subcutaneous	28	Severe symptoms
" 13, 1907	1,010	6	Horse (roan) serum	Peritoneum	29	Severe symptoms
" 11, 1907	1,011	6	Egg-white in salt solution	Subcutaneous	27	Dead 50 minutes
" 11, 1907	1,012	6	Whole milk	Subcutaneous	27	Marked symptoms
" 12, 1907	1,012	6	Egg-white in salt solution	Subcutaneous	28	Very severe symptoms
" 13, 1907	1,012	6	Horse (roan) serum	Peritoneum	29	No symptoms
July 1, 1907	1,012	6	Horse (roan) serum	Peritoneum	46	Very severe symptoms

given, usually after an interval of one day, one of the other substances used, and those withstanding it were finally tested for susceptibility to the third substance used. As will be seen from the detailed experiments, some of the animals survived after having had severe symptoms as a result of injections of all three of the proteids used. The detailed experiments and comments are given in Table 1.

From this table it will be seen that the pigs (Nos. 1,009, 1,010, 1,012) survived after having severe symptoms following the second injection of each of the three substances used. One (No. 1,011) died as a result of the injection of the egg-white, the first substance with which it was tested. One pig (No. 1,008) resisted the second injections of the horse serum and milk after severe reactions but died from the final injection of egg-white. It is therefore plain that guinea-pigs can be sensitized to horse serum, egg-white and milk at the same time, when the first injection is made in different parts of the body.

TABLE 2.

GUINEA-PIGS SENSITIZED BY PROTEID MIXTURES AT SAME TIME AND PLACE.

May 15, 1907, the following guinea-pigs received 1 c.c. whole milk, 1 c.c. saturated solution of egg-white in salt solution, and 1/250 c.c. of normal horse (roan) serum. These substances were well mixed before being injected subcutaneously into the guinea-pig. The pigs were subsequently tested as follows:

Date	No. of G.-P.	Amount	Substance Injected	Method of Inoculation	Interval in Days since First Injection	Result
June 10, 1907	1,013	6 c.c.	Horse (roan) serum	Peritoneum	26	Very severe symptoms
" 11, 1907	1,013	6	Whole milk	Subcutaneous	27	Marked symptoms
" 12, 1907	1,013	6	Egg-white in salt solution	Peritoneum	28	Dead 50 minutes
" 11, 1907	1,014	6	Whole milk	Subcutaneous	27	Marked symptoms
" 12, 1907	1,014	6	Egg-white in salt solution	Subcutaneous	28	Dead 120 minutes
" 11, 1907	1,015	6	Egg-white in salt solution	Subcutaneous	27	Dead 48 minutes
" 11, 1907	1,016	6	Whole milk	Subcutaneous	27	Marked symptoms
" 12, 1907	1,016	6	Egg-white in salt solution	Subcutaneous	28	Very severe symptoms
" 13, 1907	1,016	6	Horse (roan) serum	Peritoneum	29	No symptoms
July 1, 1907	1,016	6	Horse (roan) serum	Per eum	46	Marked symptoms
June 11, 1907	1,017	6	Egg-white in salt solution	Subcutaneous	27	Dead 45 minutes

From this series it will be seen that one guinea-pig (No. 1,016) reacted to, but survived the second injection of each of the substances used. One (No. 1,013) died as a result of the second injection

of egg-white, this being the third of the substances for which the animal was tested, the other two having caused symptoms. One (No 1,014) died following the injection of egg-white, the second substance used. Two (Nos. 1,015 and 1,017) died following the injection of egg-white, the first substance tested. From this it

TABLE 3.

GUINEA-PIGS SENSITIZED AT DIFFERENT TIMES AND IN DIFFERENT PLACES.

May 15, 1907, the following guinea-pigs were given 1 c.c. whole milk; May 29, 1907, 1 c.c. saturated solution egg-white in salt solution; and on June 12, 1907, 1/250 c.c. normal horse (roan) serum. All injections were given subcutaneously. The pigs were subsequently tested as follows:

Date	No. of G.-P.	Amount	Substance Injected	Method of Inoculation	Interval in Days since First Injection of Corresponding Proteid	Result
June 29, 1907	1,021	3 c.c.	Egg-white in salt solution	Subcutaneous	30	Dead 18 minutes
" 29, 1907	1,026	2	Egg-white in salt solution	Subcutaneous	30	Dead 50 minutes
" 29, 1907	1,027	3	Egg-white in salt solution	Peritoneum	30	Dead 15 minutes
" 29, 1907	1,019	5	Horse (roan) serum	Peritoneum	17	Very severe symptoms
July 3, 1907	.....	6	Whole milk	Peritoneum	48	Severe symptoms
" 3, 1907	.....	6	Egg-white in salt solution	Subcutaneous	34	Dead 40 minutes
June 29, 1907	1,018	6	Whole milk	Peritoneum	44	Very severe symptoms
July 1, 1907	.....	6	Horse(roan) serum	Peritoneum	19	Mild symptoms
" 3, 1907	.....	3	Egg-white in salt solution	Peritoneum	34	Marked symptoms
June 29, 1907	1,022	6	Whole milk	Peritoneum	44	Severe symptoms
July 1, 1907	.....	5	Horse (roan) serum	Peritoneum	19	Mild symptoms
" 3, 1907	.....	3	Egg-white in salt solution	Peritoneum	34	Dead 30 minutes
June 29, 1907	1,024	5	Whole milk	Peritoneum	44	Very severe symptoms
" 29, 1907	.....	6	Horse (roan) serum	Peritoneum	17	No symptoms
July 1, 1907	.....	6	Horse (roan) serum	Peritoneum	19	No symptons
" 3, 1907	.....	3	Egg-white in salt solution	Peritoneum	34	Mild symptoms
June 29, 1907	1,020	3	Horse (roan) serum	Peritoneum	17	Very severe symptoms
" 29, 1907	.....	6	Whole milk	Peritoneum	44	No symptoms
July 1, 1907	.....	6	Horse (roan) serum	Peritoneum	19	No symptoms
" 3, 1907	.....	3	Egg-white in salt solution	Peritoneum	34	Severe symptoms
June 29, 1907	1,025	6	Horse (roan) serum	Peritoneum	17	Very severe symptoms
July 3, 1907	.....	6	Whole milk	Peritoneum	48	Severe symptoms
" 3, 1907	.....	.....	Egg-white in salt solution	Subcutaneous	34	Very severe symptoms

appears that guinea-pigs can be sensitized at the same time to horse serum, egg-white, and milk, when these three substances are mixed and then injected into the animal.

This table shows that four guinea-pigs (Nos. 1,018, 1,024, 1,020, 1,025) reacted to, but survived a second injection of all three of the substances used. Two guinea-pigs (Nos. 1, 019 and 1,022) died as a result of the second injection of egg-white, this being the third of the substances with which they were tested. Three (Nos. 1,021, 1,026, 1,027) died from a second injection of egg-white, the first substance with which they were tested.

Attention is drawn especially to guinea-pig No. 1,019. This animal reacted sharply to the second injection of milk. The guinea-pig presented the usual symptoms from which it gradually recovered. *Five hours* after the injection of milk, it was given an injection of egg-white from which it died. These two reactions in so brief a time seem to accentuate the specific nature of the phenomenon we are studying. It also adds weight to our belief that profound chemical changes probably in the central nervous system explain the essential features of the reaction, rather than morphologic alterations.

TABLE 4.

The two following guinea-pigs served as control for the above three series:

Date	No. of G.-P.	Amount	Substance Injected	Method of Inoculation	Previous Treatment	Interval in Days	Result
June 11, 1907	7,841	6 c.c.	Whole milk	Subcutaneous	Tox. 9.24 + 1/330 antitox. serum Alex. 192	111	No symptoms
" 29, 1907	....	6	Horse (roan) serum	Peritoneum	.....	18	+ 45
" 29, 1907	7,848	6	Egg-white in salt solution	Subcutaneous	Tox. 9.24 + 1/300 antitox. serum Alex. 192	111	No symptoms
" 29, 1907	.....	6	Horse (roan) serum	Peritoneum	.....	18	+ 12

This table shows that guinea-pigs are not sensitized by a previous injection of horse serum to a subsequent injection of milk or egg-white. When tested against horse serum they both died.

CONCLUSIONS.

Guinea-pigs may be sensitized to three strange proteids, viz., blood serum, egg-white, and milk, at the same time. The hypersuscepti-

bility to each proteid substance is manifested by a second injection of the corresponding proteid. The three reactions are as distinct and specific as three separate infectious diseases. We, therefore, feel justified in concluding that the phenomenon of anaphylaxis is specific. We further believe that this work indicates that chemical changes, rather than morphological alterations, lie at the basis of this reaction.

STUDIES IN MENINGOCOCCUS INFECTIONS.*

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BACTERIOLOGY.

DURING the winter and spring of the last two years (1905, 1906) there appeared in various parts of the city of Chicago, a number of cases of cerebrospinal meningitis. For the most part the cases were distinctly sporadic and such cases coming to the Cook County and Presbyterian Hospitals of this city afforded an opportunity for this study.

The chief points obtained from the bacteriologic examination are given in tabular form (Table 1). Of 11 cases, 10 occurred in men. The age varied from 3 to 32, six being between 10 and 20 years. For the most part they were foreigners living in the poorer parts of the city. Four of the 11 died. Diagnosis was made in all cases by lumbar puncture and the meningococcus of the Weichselbaum type isolated in every case. In smears made from the cerebrospinal fluid it was found in all cases but one, and this was a very mild case. Without exception the cerebrospinal fluid was under pressure and was turbid. Upon standing from 15 to 30 minutes a delicate clot of fibrin formed as a rule more abundant in the severer cases than in the milder ones, and also more abundant early in the disease than later. The fluid after the cells and fibrin were centrifugated out was usually clear and colorless. In one case it had a distinctly greenish tinge. This was during the third week and occurred in a mild case. Flakes of fibrin, white or yellowish in color, were occasionally obtained but never any blood clots. The fluid when a free flow was obtained, always became practically colorless after the blood due to the puncture had been washed away. Uncoagulated blood was not present in any instance. Hemolysis in the fluid was never encountered. Differential counts of the leucocytes were not made but the polynuclear leucocytes were by far the predominating cell, there being always a few mononuclears and large cells of the endothelial type. In one case examined late in the disease (fourth week) the latter cells were very numerous.

The diplococci were found both inside and outside the polynuclear leucocytes and never inside any other cell. The number varied considerably, being on the whole more numerous in the severe cases than in the milder. There was considerable variation in the numbers of cocci in different cases and in the same case from time to time. As a rule, the less severe the disease the greater the number of cocci found inside the cells, but this is only generally true and exceptions occurred. During convalescence especially, the cocci were nearly always found more completely englobed by the cells than earlier in the disease.

The organisms corresponded in every instance to the Weichselbaum type of meningococcus. All were Gram-negative and showed no appreciable variations in

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TABLE 1.

BACTERIOLOGY OF CASES OF EPIDEMIC CEREBROSPINAL MENINGITIS.

No.	Sex	Age	Nationality	Meningococci in Cerebrospinal Fluid		Meningococci on Nasal Mucosa		Blood	Eye	Agglutination	Termination
				Smears	Cultures	Smears	Culture				
1	M	19	Italian	++Chiefly outside leucocytes	+	Gram-negative diplococci	30%	−	....	−	Death
2	M	19	Italian	++Mostly outside leucocytes	+	Gram-negative diplococci	0	0	....	+1:50	Recovery
3	M	A[1]	Italian	+	+	Gram-negative diplococci	0	0	....	−	Death
4	M	16	Italian	+	+	Gram-negative diplococci	0	0	....	+1:50	Recovery
5	M	26	Italian	0	+	No Gram-negative diplococci	0	0	....	+1:100	Recovery
6	M	32	Irish	+Chiefly inside leucocytes	+	Not examined	Not examined	−	....	−	Recovery
7	M	10	German	++Mostly outside leucocytes	+	Gram-negative diplococci	+90%	+	....	+1:50	Death
8	M	3	German	++Numerous; chiefly inside the leucocytes	+	Gram-negative diplococci	+90%	+	Purulent conjunctivitis Men.+, nearly pure	+1:100	Recovery
9	M	30	Bulgarian	+Largely inside leucocytes	+	Gram-negative diplococci	+ 5%	0	Conjunctivitis. Meningococci negative	+1:500	Recovery
10	F	18	German	Not examined	+	Not examined	Not examined	0	....	+1:50	Death
11	M	13	American	+ Few cocci outside of the leucocytes	+	Not examined	Not examined	0	....	+1:50	Recovery

[1] A = Adult.

this respect. For cultivating the cocci the spinal fluid was usually added to ascites broth (1:4) which was found suitable and reliable for this purpose. In 24 hours, growth is manifested by a marked tubidity. Broth to which a small quantity of defibrinated blood has been added also makes a good medium. Blood-agar, Löffler's blood serum and calcium broth are all satisfactory. The cultures must be transferred every three or four days or preferably every other day. At the end of four or five days they often die. Cultivation of several strains for over four months, transfers being made on Löffler's blood-serum every three or four days and often more frequently, did not show any appreciable alteration in the cultural or morphological characteristic except, perhaps, a little more vigorous growth. For plating ascites-glucose-agar (1:4) and blood agar (10 drops in 5 c.c. of glucose-agar) are good media. The colonies in 24 hours are 0.5 to 1 mm. in diameter with sometimes larger surface colonies. They are of a delicate bluish gray and do not hemolyze. When grown on plates they give off a peculiar odor difficult to describe but suggesting moldy earth. This is always obtained but does not appear to be characteristic. On blood serum one frequently sees a very profuse growth like a giant colony 2 or 3 mm. in diameter decidedly raised above the surface. This may be one large colony or may be formed by the fusion of several colonies. It appears to be quite characteristic of the meningococcus. The growth especially on blood serum is often tenacious and when suspended in salt solution or water appears as flakes and threads which may be broken up by vigorous agitation. Strains vary in this property, it being evident in all, but in many it is much more conspicuous than in others. It differs decidedly in this respect from *M. catarrhalis*, whose growth is quite brittle, is readily broken up, and never tends to form flakes when suspended from blood serum in salt solution.

Examination was made of the nasal secretion in eight cases. In smears made directly from the mucosa, Gram-negative diplococci morphologically suggestive of the meningococcus were observed in seven of the eight cases. In four cases meningococci were isolated on blood-agar or ascites-agar plates and in two of these (Cases 7 and 8) they formed approximately 90 per cent of all organisms present in the plates. In Case 1 they formed about 30 per cent and in Case 9 about 5 per cent. In the smears made from the mucus the Gram-negative diplococci were very numerous, and it appears, from a comparison of the plates and the smears, that often a large number of the cocci occurred which were dead or did not develop. There is little doubt that the Gram-negative organisms seen in nearly all the smears were meningococci, though, so far as one could tell from their morphology, they may have been *M. catarrhalis*. Among other organisms obtained, besides the commonly found Gram-positive diplococci of the pneumococcus type may be mentioned the occurrence in five out of the eight cases examined, of hemophilic bacteria resembling the organisms found in measles, whooping-cough, bronchitis, influenza, and other infectious diseases. In some of the cases they were very abundant and, it is possible, played a part in producing the nasal symptoms.

In two cases (Cases 8 and 9) a conjunctivitis existed early in the disease. In Case 8 there was a purulent discharge, smears of which showed some Gram-negative diplococci. Cultures gave a nearly pure growth of typical meningococci. The conjunctivitis was not severe and in a few days (under boric acid irrigation) cleared up, leaving no lesion. In Case 9 smears and cultures from the conjunctiva showed almost pure growth of a bacillus of the diphtheria group. No meningococci were present. This infection likewise cleared up in a few days.

Blood cultures were made in 9 cases with two positive results. This is about 22 per cent or about the same as found by Elser[1] in New York. In both of these cases (7 and 8) positive results were obtained within the first four days of the disease. Case 7 died on the fifth day and Case 8 recovered. In some of the cases cultures were not obtained until after the first week. I feel quite confident that if cultures of the blood, using large quantities, were made very shortly after the onset of the attack and at frequent intervals thereafter the percentage of positive results would be much higher. In Cases 2 and 9 both severe, though both ultimately recovered after a long period, cultures were made at three different times at intervals of a few days, always with negative results. That the presence of the meningococcus in the blood does not necessarily mean a fatal result is shown by the result in Case 8.

Herpes was present in nearly all the cases. In two, several cultures were made from the contents of the vesicles at different stages. Staphylococci were always obtained but no meningococci.

Case 8 shows the remarkable extent to which invasion of the body by meningococci is possible and recovery ensues. The organisms in this case were cultivated from the eyes, nose, blood, and cerebrospinal fluid. On the nasal mucosa they were very numerous. From the blood an abundant growth was obtained by introducing 1 c.c. in ascites broth. During the third week of the disease a double otitis media developed. Cultures and smears were made from the discharge on the first and fifth days after it appeared and at the same time cultures were made from the nasal mucosa. At this time a diphtheria-like bacillus was obtained from the nose and a mixed growth of this bacillus and streptococci found in both ears. No meningococci were found. This is evidently therefore an instance in which a primary meningococcus infection furnished suitable conditions for the secondary invasion of other organisms, a process so commonly seen in other acute infections.

The manner and order in which the meningococcus gains access to the various tissues of the body is a problem of importance but one difficult to solve. That many cases of meningitis are associated with bacteria in the blood is established. Since it is an organism much more difficult to cultivate than the typhoid bacillus or pneumococcus, we can hardly expect to obtain the meningococcus from the blood in all the cases where it is present. Other evidence of the invasion of the body by the meningococcus is localization of the organism in the pericardium, in various joints, and in other places. In this series there occurred a purulent pericarditis in one case. Pure meningococcemia with no definite localization elsewhere than in the meninges is rare; a few cases are reported. Cases have been described also in which the organism has been recovered from the blood and from joint lesions some time before the appearance of meningeal symptoms, thus indicating a primary blood infection with secondary localization in the meninges. These observations naturally bring

[1] *Jour. Med. Res.*, 1905, 14, p. 89.

up questions as to the mode of entry of the meningococcus into the system.

In view of the relatively frequent occurrence of the meningococcus on the nasal mucosa this locality may be an important starting-point in its invasion of the body. As was first suggested by Weichselbaum, the frequent preceding and coincident acute rhinitis makes this view seem reasonable, the inflamed mucosa allowing the cocci to pass directly into the blood vessels and lymph spaces. There is also the possibility, as Westenhoeffer has pointed out, that the throat and tonsils may be portals of entry since they not infrequently are involved early in the disease. The meningococcus is often found in abundance in the throats of patients. Probably the mucosa of the whole respiratory tract should be looked upon as at times affording a suitable point of entrance for the meningococcus. It is improbable that the gastro-intestinal tract is a portal of entry for the cocci because they are so sensitive to acids.

It is commonly supposed that the organisms, after passing through the mucosa, enter the lymphatics and make their way directly through the base of the skull to the meninges. However, it does not necessarily follow that because the point of entrance and the localization of infection are near each other the organism reaches the latter place by the shortest possible route. The predilection for localization upon the meninges may be analogous to what occurs in other infections. Thus, intravenous injection of cholera bacilli into animals is followed by a localization of the infection in the small intestine. Dysentery becomes localized in the large intestine. Gonococci localize generally upon serous surfaces, especially of the joints. Tubercle bacilli enter through the respiratory or intestinal mucosa and often localize in some remote part of the body, causing no lesion at or near the place of entry. So meningococci, when once in the blood or lymph, may be carried throughout the whole body and only secondarily, because of strong affinity for the meninges, settle there. Occasionally, as in other diseases, an anomalous localization occurs, such as in a joint; or localization may fail to take place and a meningococcemia results.

While the anatomical relations of the nasal mucosa and the meninges may play some part in determining the site of the lesion, an even more important factor would seem to be the peculiar affinity of the organism

for the meninges. The nature of this affinity is not known, but it conforms to a principle common in infectious diseases and emphasizes that the chemistry of the structures may be of greater importance than anatomical relations in determining the localization of an infective process.

The discussion of the bacteriology of meningitis naturally raises the question of the manner in which the organisms are distributed and how sporadic cases arise. The large numbers of cocci in the secretions of the nose and throat of patients, show how easily the cocci may be transferred by coughing, sneezing, breathing, etc. Their occurrence in the nose and throat of persons associated with meningitic patients which has now been demonstrated repeatedly, is sufficient evidence that the cocci are easily transferred, and emphasizes the importance of careful isolation of cases and proper disinfection of the throat and nasal cavities of persons closely associated with patients.

Sporadic cases, however, present problems of their own. They are identical with those in epidemics, the organisms from both groups corresponding in every respect. The meningococcus is so delicate that it does not seem possible that it can live over, at the most, a few days outside of the human body. Animals, so far as we know, are naturally immune and do not harbor the coccus. There are, therefore, only two possibilities to explain sporadic cases. The first is that the cocci are carried from person to person without causing symptoms until, perhaps, after many transfers an individual is encountered in whom the conditions are favorable for their development and entrance into the system. If this be true, one would expect to find meningococci, occasionally at least, in the throats and noses of individuals, normal or abnormal. This, however, does not appear to be true from the data at hand. In over 200 examinations, made by the writer, of the sputum and throats of more than 150 individuals, by means of the blood-agar plate method, the meningococcus was never found. Others have made careful bacteriological examinations of the throat in health and disease but report no meningococci. Hasslauer[1] studied 192 cases with especial reference to the meningococcus and concludes that this organism occurs only in the throat of men-

[1] *Centralbl. f. Bakt.*, 1906, 41, p. 633.

ingitic patients and of persons in their immediate vicinity. However, this does not prove absolutely that the meningococcus does not occur occasionally in the normal throat, for if only occasionally present in few numbers it might easily elude detection. It does show, however, that the meningococcus, unlike the pneumococcus and streptococcus, if it occurs at all, must do so infrequently and then probably in small numbers; otherwise it would surely have been detected.

The other possibility, which is remote, is that closely related organisms, commonly found on the respiratory mucosa, may be modified under certain conditions and acquire all the properties of the meningococcus. There is a group of Gram-negative diplococci, including *M. catarrhalis*, found in the nose and throat, some of which closely resemble the meningococcus but yet possess well-marked points of difference in cultural characteristics and especially in agglutinating properties. Such transition has not been observed, and is as yet a theoretical possibility.

PROPERTIES OF THE BLOOD AND SERUM IN MENINGITIS.

Agglutination.—Agglutination tests were made in eight cases. In all a positive result was obtained in dilutions of 1:50. Both microscopic and macroscopic methods were used. The highest dilution at which agglutination occurred was 1:500 (Case 9). This was at the beginning of the fourth week of the disease. At the end of the first week the serum agglutinated strongly at 1:25, at 1:50 slightly; in the eighth week the agglutination occurred at 1:200 and not at 1:500. This case recovered after a protracted course. In two other cases the agglutination occurred at a dilution of 1:100. A distinct reaction was not obtained in the other cases higher than at 1:50.

The agglutination test in Case 8 gave negative result on the fifth day after the first evidence of any symptoms, or the third day after distinct meningeal symptoms had appeared; it was obtained with an organism isolated from another case. Two days later agglutination appeared at a dilution of 1:25 with this same organism and also at the same dilution with meningococci isolated from the blood, cerebrospinal fluid, nose, and conjunctiva of the case itself. Later the serum agglutinated at a dilution of 1:100. In two other cases agglutination

was obtained on the seventh day. From the above data we see that agglutination is obtainable at the end of the first week, approximately.

The meningococcus agglutinins are thermostable bodies resisting, with little diminution in their activity, a temperature of 65° C. for one hour.

The cerebrospinal fluid does not appear to contain the agglutinins in an appreciable amount. In Case 9 the pure fluid gave practically no evidence of clumping, while the serum of the patient removed at the same time produced marked agglutination at 1:50 or higher. This test was made at two different times and both homologous and heterologous strains of organisms were used. I have also looked for clumping of the cocci in the fluid as it comes from the canal in cases in which the serum gave a positive test at 1:50 or higher, but always with negative results.

A rabbit was immunized with a typical meningococcus isolated from the spinal fluid of Case 6. Subcutaneous inoculations were first used, followed by intraperitoneal and intravenous. The animal decreased in weight and lost much of its hair; it became immune to large doses of meningococci, easily resisting 10 times the dose that at first made the animal quite ill. A number of strains isolated from various sources were tested and the result is given in Table 2. The

TABLE 2.

AGGLUTINATION OF MENINGOCOCCI BY IMMUNE RABBIT SERUM.

Source of Organism	1:20	1:50	1:100	1:200	1:500	Salt Solution
1 Cerebrospinal fluid (Case 9)	++	+	o	o	o	o
2 Nasal Mucosa (Case 9)	++	++	o	o	o	o
3 Cerebrospinal fluid (Case 6)	++	++	o	o	o	o
4 " " " (Case 8)	++	+	o	o	o	o
5 Nasal Mucosa (Case 7)	+++	++	o	o	o	o
6 Conjunctiva (Case 8)	++	+	o	o	o	o
7 Blood (Case 8)	+++	++	o	o	o	o
8 *M. catarrhalis*	++	o	o	o	o	o

homologous organism was agglutinated at a slightly higher dilution than the other organisms, but the difference is not great. The serum of patients also shows no special difference in its agglutinating power over different strains.

M. catarrhalis (isolated from a case of whooping-cough) showed less clumping than the meningococci. The controls with normal rabbit serum gave negative results or only very slight clumping at 1:10.

It may be stated that all the meningococci isolated from the blood, nose, and eyes of the cases reported were tested either with immune rabbit serum or with serum of patients, and that all, without exception, reacted positively. They are therefore considered typical meningococci.

Antimeningococcal properties of blood and serum.—In a previous paper[1] it was shown that meningococci, when introduced into normal defibrinated blood, as a rule would not multiply. In some normal bloods, after an initial drop, the cocci multiplied rapidly, thus indicating interesting individual variations. In the blood of several meningitic cases tested at varying times from the tenth day of the disease to the seventh week, the meningococci without exception were rapidly killed, the plates usually being sterile at the end of three hours. It was also found that in the serum of both normal persons and meningitic cases the meningococci rapidly die, but that the meningococcidal effect of the meningitic serum is greater than that of the normal. Furthermore, the destructive effect of the serum is much greater than that of normal salt solution so that, while the cocci tend to die out rapidly as a result of autolytic processes, there can be no doubt but that serum exerts a distinct lytic effect on meningococci, and this effect is greater in meningitic serum than in normal.

In Table 3, the meningococcidal effect of meningitic serum in the fourth week is given. The serum was tested by the plate method in

TABLE 3.

EFFECT OF MENINGITIC SERUM ON MENINGOCOCCI.

		At once	3 Hrs.	6 Hrs.	24 Hrs.
1	Nor. Ser. 0.4	6,000	0	0	0
2	" " 0.3+asc. br. 0.1	6,000	51	0	0
3	" " 0.2+ " " 0.2	4,600	166	176	500
4	" " 0.1+ " " 0.3	5,000	222	140	9,000
5	Men. Ser. (4 W.) 0.4	5,000	0	0	0
6	" " " 0.3+Asc. br. 0.1	4,000	16	0	□
7	" " " 0.2+ " " 0.2	5,500	67	3	□
8	" " " 0.1+ " " 0.3	6,000	220	88	2,500
9	Ascites Broth	6,000	4,500	7,000	10,000

two cases early in the disease, one on the fifth day and the other on the sixth. In both cases (7 and 8) the organism was cultivated from the same blood from which the serum was obtained for the experiment. In each case the meningitic serum was slightly less bactericidal than

[1] *Jour. Infect. Dis.*, 1905, 2, p. 602.

the normal serum; but the difference is so slight that one is hardly justified in considering it of great significance. The agglutination test in one case gave negative results at the time; in the other case the test was not made until two days later when it was positive at 1:25.[1] In Case 7, also on the fifth day, the meningococcidal effect of the defibrinated blood, in which meningococci were found by cultures, was tested and in this case the cocci grew. This was only a short time before death; the leucocyte count was 22,000. We see then that late in the attack the serum acquires definite meningococcidal properties. The subject of phagocytosis of meningococci is taken up elsewhere and here it is sufficient to state that the cocci are readily taken up by the leucocytes in defibrinated blood and very soon undergo rapid disintegration within the cells.

From some early experiments it did not appear that the meningococcidal property of the blood ran parallel with the leucocytosis, but was found to be increased later in the disease when the leucocytic count was practically normal. And, as stated, meningococci grew in defibrinated blood in one case when the leucocytosis was high (22,000). Samples of blood containing varying quantities of leucocytes were obtained by centrifuging and using the top and bottom layers, the former containing many leucocytes and the latter very few. This blood was now heated to 46° C. for 10 minutes, so as to destroy phagocytosis but not to injure the action of complement. The results are

TABLE 4.

EFFECT OF LEUCOCYTES ON MENINGOCOCCI.

		Leucocyte Count	At once	3 Hrs.	6 Hrs.	24 Hrs.
1	Defib. men. blood 0.4 c.c.....	30,000	1,600	0	0	0
2	" " " 0.4	11,200	6,000	0	0	0
3	" " " 0.4	7,100	1,500	0	0	0
4	" " " 0.4	4,000	1,000	0	0	0
5	" " " 0.4	600	5,400	4	0	0
6	" " " 0.4	4,000	5,000	2	0	0
	(Heated to 46° C. 10 min.)					
7	" normal blood 0.4 c.c.....	4,400	1,100	37	1,800	15,000+
8	" " " 0.4 c.c.....	4,400	2,000	111	10,000	6,000
	(Heated to 46° C. 10 min.)					

[1] The point may be raised that the apparent meningococcidal action, as shown by the plate method, is due to the agglutination of the organisms. I have no doubt but that in agglutinating sera the clumping may explain at least part of the diminution in the number of colonies appearing on the plates at various intervals. However, this does not alter the fact that the bacteria are killed, as shown by the result after several hours, and it is immaterial to us in this present consideration of the question whether part of the bactericidal action, as shown by this method, is due to agglutinins or all due to other factors, such as complement action or an enzymotic process.

given in Table 4, and we see that the number of leucocytes in the blood has little if any effect on the organisms so far as results on plates indicate. In one experiment blood containing 600 leucocytes was just as fatal as one containing 30,000.[1] In a normal blood, in which the cocci readily multiplied, the killing of the leucocytes by heat made no difference in the rapidity of multiplication. It is difficult to understand why the effect of phagocytosis is not more apparent; it is possible that it is overshadowed by the stronger action of the serum.

As shown previously meningococci do not grow in cerebrospinal fluid, either normal or meningitic. *In vitro* one cannot obtain the same conditions as are present in the spinal canal. After leaving the canal fibrinogen, for instance, is soon transformed into fibrin, i. e., changed from a soluble to an insoluble compound. Such changes in the properties may explain why the cocci grow profusely in the canal and rapidly die in the test tube. Serum may be added to cerebrospinal fluid or salt solution in any proportion; the organisms nevertheless die rapidly. If red corpuscles, however, are added to the fluid an excellent medium is obtained. This indicates that the blood in the cerebrospinal fluid may facilitate greatly the growth of meningococci.

There are then several factors concerned in the development of meningococci. In the first place the organism is extremely sensitive and, as we have seen, dies in salt solution in a few hours. Flexner[2] recently concludes that this is largely due to autolysis. In serum the cocci may be observed directly to undergo a rapid disintegration. While this process of autolysis probably goes on in serum, as it does in salt solution, there is also another factor which makes the serum much more unfavorable than salt solution. This may be an amboceptor-complement action and the increased meningococcidal power of meningitic serum may be due to an increase in the amboceptor during the course of the disease. Phagocytosis we cannot doubt also plays a part in the destruction even though it is not clearly brought out by the plate method. Opposed to these destructive processes there are other conditions in blood directly favoring growth, probably the most

[1] The amount of serum in the tubes was as nearly as possible the same.

[2] *Jour. Exp. Med.*, 1907, 9, p. 105.

important of which is the presence of red cells or hemoglobin. Of course other proteids of the blood may favor growth also. Serum, for instance, which in high concentrations is deadly, favors growth when sufficiently diluted, as shown by adding serum to plain broth, in which meningococci do not grow at all or very poorly; yet when the serum is diluted several times by the broth a favorable medium is formed. Here the antagonistic effects of the serum when concentrated is superseded by its nutritive properties.

Thus we find two sets of factors, one favoring the multiplication of meningococci, the other destructive. Infection therefore with this organism and the character of its subsequent course must depend upon the relation which these two sets of factors bear to each other in the various tissues in the body, and especially in the blood and the cerebrospinal fluid. It is evident from the data given upon the effect of blood on meningococci that in this substance these factors are almost in a state of equilibrium. Sometimes the cocci grow and sometimes they do not. No great change is required to upset this adjustment. There are no definite data at hand bearing upon changes in these factors that may precede an attack of meningitis. During the course of the disease, however, we note a definite change in favor of the host and against the invading microbe, namely an increased meningococcidal property of the serum, due perhaps to an increase in amboceptor, and, as is shown subsequently, in an increase in the opsonic content of meningitic serum as compared with normal serum. When we consider the delicate balance of various factors in normal blood it is probably true that changes even so small as to be within the present limits of experimental error may be quite sufficient to bring about a destruction either of the host or of the microbe.

Phagocytosis.—As shown previously[1] meningococci are readily taken up *in vitro* by leucocytes in the presence of serum, but practically not at all by washed leucocytes or by leucocytes in the presence of normal serum heated to 60° C. for 30 minutes. The number of cocci taken up by the leucocytes is roughly proportional to the amount of serum present as shown in Table 5.

In view of the fact that the opsonic content has been found to be increased in some acute infections one naturally would look for a

[1] *Jour. Infect. Dis.*, 1905, 2, p. 602.

TABLE 5.

PHAGOCYTOSIS IN NORMAL AND MENINGITIC SERA.

										Normal Serum	Men. Ser. 10th Day (Case 8)	Men. Ser. 2d Wk. (Case 9)
1	Washed Corp.	0.2 c.c.	+	Ser.	0.2	+	Susp. cocci.		*0.2...	6.1	8.0	7.6
2	" "	0.2 c.c.	+	"	0.1	+	" "		0.2...	2.9	6.1	4.5
3	" "	0.2 c.c.	+	"	0.05	+	" "		0.2...	2.6	4.7	3.1
4	" "	0.2 c.c.	+	"	0.025	+	" "		0.2...	1.6	2.4	2.1
5	" "	0.2 c.c.	+	"	0.0125	+	" "		0.2...	1.6	2.4	1.6
6	" "	0.2 c.c.	+	"	0.00625	+	" "		0.2...	0.4	1.0	0.9

*The organism used in this experiment was obtained from the cerebrospinal fluid of Case 8. Note the greater phagocytosis in the homologous serum. In each case the total quantity was brought up to 6 c.c. and the mixtures incubated at 37° C. for 10 minutes. The number of cocci given represents the average within 100 leucocytes.

similar increase in meningitis. In my previous paper I stated that by the method used no appreciable increase was observed in the opsonin in the few cases then studied.

Since then I have made a number of experiments using falling quantities of serum and testing the blood from time to time in individual cases. While many of the experiments were unsatisfactory—because of certain difficulties referred to later the results on the whole show somewhat greater phagocytosis in meningitic serum than in normal serum (Table 5). Other determinations did not show as great

TABLE 6.

PHAGOCYTOSIS OF MENINGOCOCCI IN MENINGITIC AND NORMAL SERA.

	Source of Organism	Meningitic Serum (Case 9) 8th Wk.	Meningitic Serum (Case 10) 4th Wk.	Normal Serum
1	Cer.-sp.fluid (Case 10).....	4.6	4.4	2.2
2	" " " (Case 9).....	5.0	8.3	2.4
3	Blood (Case 8).....	5.8	7.7	4.3
4	Nasla mucosa (Case 7).....	9.8	13.8	9.7
5	Cer.-sp. fluid (Case 11).....	4.4	5.1	4.9
6	" " " (Case 8).....	8.1	11.8	8.0
7	Conjunctiva (Case 8).....	14.0	16.5	7.5
8	Cer. Sp. fluid (Case 9).....	3.0	4.8	3.5
9	Nasal mucosa (Case 9).....	8.2	10.2	9.8
	TOTAL.....	62.9	82.6	52.3
	AVERAGE	7.0	9.2	5.8

a difference between normal and meningitic serum and others showed a greater. Table 6 gives the results obtained with normal serum and the serum of two meningitic patients, one of whom had been sick about four weeks and the other eight. Nine strains of meningococci from various sources were used. Some strains show a marked difference of phagocytosis in favor of the meningitic sera, others very

little or none at all. On the whole the serum from the patient in the fourth week shows a marked increase. If the numbers indicating the phagocytosis for each serum are added together and the average determined, the amount of phagocytosis is seen to be decidedly greater in the cases of the meningitic sera. The same suspensions of organisms were used for each serum so that the results are comparable. In order to obtain the exact course of the meningococco-opsonic index in meningitis daily observations should be made throughout the attack.

If normal cerebrospinal fluid is added to washed corpuscles no phagocytosis results, and the same seems true when the fluid of meningitic patients is used; at all events the amount of phagocytosis is very slight. The fluids from two cases gave practically the same result. In each instance the fluid before centrifugation contained many meningococci and leucocytes, some of which contained organisms. If counts were made of the phagocytosis *in vivo*, the count on the whole would be low, for many of the leucocytes are disintegrating and the cocci are few in numbers as a rule. We may conclude that normal cerebrospinal fluid has practically no opsonin and the meningitic fluid has a small but insignificant amount as compared with that in blood serum. It probably does not require much opsonin in order to give the phagocytosis that occurs in meningitic fluid, because cocci are exposed to the opsonin for a relatively long period of time under favorable conditions.

In regard to the susceptibility to phagocytosis of different strains of meningococci there does not appear to be a marked difference. They are susceptible to phagocytosis immediately after isolation from the spinal fluid and from the blood, and are apparently as freely taken up at this time as after cultivation (Table 6). All strains are susceptible, but the figures as given are not comparable because of the varying concentration of the suspensions. Since the organisms are so freely taken up, slight differences in susceptibility to phagocytosis, which may mean a difference in virulence, is difficult to detect. Strain 1, for instance, was an organism isolated from the cerebrospinal fluid of a fatal case and had been under cultivation only a few days. It was highly toxic for man as shown by subsequent subcutaneous injection. No. 8 was an organism isolated from the cerebrospinal

fluid of a very mild case and had been under cultivation for several months, being transferred to fresh media every two or three days. There seems to be little difference in the susceptibility of these two organisms to phagocytosis. One sometimes observes strains which apparently are not taken up or seemingly much less so than others. This I have found to be explained by the fact that many of the organisms of some cultures stain not at all or very poorly and irregularly, and consequently the counts are unreliable.

It was said that on the whole there was less phagocytosis observed in the spinal fluid of severe cases than of milder. This may mean a slight difference in virulence; but it may also be explained by a slight difference in opsonic content of the fluids, or possibly by difference in the activity of the leucocytes. In this respect the meningococcus is in marked contrast to the pneumococcus which, as shown by Rosenow,[1] is nearly or wholly insusceptible to phagocytosis when isolated from the blood of pneumonic patients. It is true that generally meningococcus meningitis is not so virulent or so fatal an infection as pneumococcus meningitis. In the latter the leucocytes contain practically no organisms though as a rule the pneumococci are extremely abundant in the fluid outside the cells. In three cases, the fluids of which I had an opportunity to examine and compare with that of meningococcus meningitis, the organisms in all were very much more numerous in the fluid than in any of the meningococcus infections studied, and practically no phagocytosis of pneumococci had occurred.

If serum is added, either to washed leucocytes in blood or to the washed leucocytes of freshly drawn meningitic fluid, the number of meningococci taken up is much increased. Two experiments with leucocytes from the spinal canal, to which serum was added showed a rise in one case from 1.1 to 3.1 and in the other from 1.2 to 8.0. From these results it would seem rational to inject human serum into the spinal canal of patients in order to increase the destructive action of the leucocytes.

Two cases were thus treated. The first case was one of only moderate severity running an ordinary course with intermittent fever. During the third week a spinal puncture was made, about 10 c.c. of a turbid fluid withdrawn and 8 c.c. of normal fresh human serum was

[1] *Jour. Inject. Dis.*, 1907, 4, p. 285.

injected in its place. There was no reaction and the patient made an uneventful recovery. One cannot say that the injection had any effect whatever, except that it did no harm.

The second case was very severe. The onset occurred with a chill and vomiting, and the temperature ranged from 104 to 106° F. There was present opisthotonus, marked rigidity of the neck, Kernig's sign, and delirium. Cultures from the nose and blood showed meningococci. The spinal fluid on the fourth day after onset was turbid, contained much fibrinogen and abundant typical meningococci chiefly outside the leucocytes. On the fifth day in the evening lumbar puncture was made and 35 c.c. of turbid fluid under considerable pressure were withdrawn and 10 c.c. of normal human fresh serum were then slowly introduced. The next morning, about 12 hours later, only 1 c.c. of turbid fluid could be obtained. There were present many leucocytes and they contained few diplococci within them. Five c.c. of fresh serum were again injected. The child gradually became worse and died on the seventh day. The serum did not have any apparent effect on the course of the disease. From the smears of the spinal fluid made 12 hours after the injection of the serum one could observe no appreciable increase in phagocytosis.

The difficulties in the way of this treatment are the failure of the serum to reach the meninges of the cord and brain and the large amount of fluid in the canal making the dilution so great that a small amount of serum could have little effect either on phagocytosis or on bacteriolysis.

Hemagglutinins and hemopsonins in meningitis.—In the phagocytosis experiments it was noted that a distinct clumping of corpuscles frequently occurred. A number of tests was made to determine if the agglutination of red corpuscles by meningitic sera was in any way characteristic.

The results show that while agglutination of red corpuscles from various sources is a common property of meningitic sera it is not specific, and sera from different cases behave differently toward the same red corpuscles. In three cases meningitic serum did not clump homologous corpuscles. It clumped some normal red corpuscles and not others. It may or may not clump meningitic red corpuscles or corpuscles from other infections as scarlet fever and pneumonia.

No changes were observed during the course of the disease or later in the agglutinative property toward various corpuscles.

The agglutinins are absorbed *only* by corpuscles which are agglutinated and may be completely removed from the serum by contact for a few hours. The agglutinin cannot be recovered by washing the clumped corpuscles in salt solution. The hemagglutinin and bacterial agglutinin present in meningitic sera were shown to be independent bodies by the fact that the serum freed from hemagglutinin by long contact with red corpuscles still retained unimpaired its power to agglutinate meningococci. Ascites broth in which meningococci are grown for varying periods (24 hours, 48 hours, one week, and two weeks) at no time show the slightest trace of hemagglutinin. Several strains of meningococci were used. The hemagglutinins are fairly stable bodies. Heating to 65° C. for 30 minutes diminishes the agglutinative power of serum but does not completely destroy it. Exposure to sunlight slowly destroys the property; but much more rapidly in the presence of eosin. The above data indicate that the hemagglutinins found in meningitic blood are not different from those occurring in normal blood; they possess no specific properties. My results agree in every respect with those of Hektoen[1] and others.

Phagocytosis of red corpuscles was observed in two meningitic sera. It was obtained by adding the sera to washed normal corpuscles and allowing to stand for one hour. The serum in one case (Case 8) was obtained on the seventh day of disease. In the other case (Case 11) the serum was obtained in the second week. The corpuscles were strongly agglutinated by the sera. Sera from two other cases of meningitis were repeatedly tested for hemopsonins always with negative results. They were strongly agglutinative for certain red corpuscles, but no phagocytosis of the corpuscles was observed at any time.

THE INJECTION OF MENINGOCOCCI INTO ANIMALS AND MAN.

It is well known that meningococci have only a small degree of virulence for the lower animals. Large doses of these organisms, either dead or alive, may be given to animals subcutaneously or intravenously with little effect; and it has been questioned whether or not the living organisms multiply at all after injection into the animals.

[1] *Jour. Infect. Dis.*, 1902, 4, p. 297.

I have injected 24-hour growths from two blood-serum slants directly into the veins of a small monkey with not the least effect. Flexner[1] has recently produced lesions in monkeys by intraspinal injections of meningococci, but he is not sure that multiplication, at least to any great extent, occurred. His results also show how insusceptible animals are to subcutaneous injections.

I have repeatedly injected rabbits subcutaneously with growths from five to ten tubes of blood serum with practically no effect. Intraperitoneal injections are more effective, but even here the lower animals can tolerate large doses of meningococci, as compared with many other organisms, as for instance virulent streptococci. Strains vary in their virulence to a certain extent, as Flexner has pointed out.

To show the result of successive inoculations into animals the following experiment was made. A typical meningococcus isolated from the cerebrospinal fluid of a non-fatal case was used.

Guinea-pig 1.[2]—Inoculated intraperitoneally with growth from one blood-serum slant. Dead in 12 hours. Peritoneal cavity contained a gelatinous non-purulent exudate in which meningococci were very numerous, leucocytes very few, and there was very little phagocytosis. Cultures from heart's blood and peritoneal exudate gave abundant meningococci.

Guinea-pig 2.—One-half c.c. of peritoneal exudate from Pig 1 was injected intraperitoneally. Next morning the animal was lively, showing no ill effects whatever.

Guinea-pig 3.—Growth from one blood-serum slant obtained from peritoneal exudate of Pig 1 was inoculated intraperitoneally. Dead in 12 hours. Peritoneal cavity contained a non-purulent, fibrinous exudate. Meningococci very numerous and leucocytes few. Meningococci in culture from heart's blood and exudate.

Guinea-pig 4.—Two and one-half c.c. of peritoneal exudate from Pig 3 injected into peritoneal cavity. In a few hours the animal was very sick and died in 12 hours. Peritoneal cavity contained a fibrinous exudate with many meningococci and few leucocytes. Cultures positive.

Guinea-pig 5.—One-half c.c. of peritoneal exudate from Pig 4 injected intraperitoneally. Dead in 12 hours. Peritoneal cavity contained a fibrinous exudate with many cocci and few leucocytes. Cultures positive.

Guinea-pig 6.—One-half c.c. of peritoneal exudate from Pig 5 injected intraperitoneally. Dead in 10 hours. Cultures from heart's blood and peritoneum gave meningococci. Peritoneal exudate contained few leucocytes many of which were filled with cocci.

Guinea-pig 7.—One-half c.c. of peritoneal exudate from Pig 6 injected intraperitoneally. In three hours the animal was very sick Died in 10 hours. Meningococci recovered in pure culture from heart's blood and from peritoneal exudate. Few leucocytes present.

[1] *Jour. Exp. Med.*, 1907, 9, p. 42.

[2] The guinea-pigs were all small, weighing from 250 to 300 grams.

Guinea-pig 8.—One-half c.c. of peritoneal exudate from Pig 7 injected intraperitoneally. Dead in 12 hours. Pure culture of meningococci obtained from heart's blood and peritoneal exudate.

Guinea-pig 9.—One c.c. of peritoneal exudate which had been heated to 65° for one hour[1] was injected intraperitoneally. No immediate effect. Next day animal appeared a little weak and not so lively. On second day the animal was normal.

Guinea-pig 10.—One c.c. of heart's blood from Pig 8 injected intraperitoneally. No effect.

Guinea-pig 11.—Injected intraperitoneally with one slant growth from Pig 8. Animal dead next morning. Usual findings in peritoneal cavity and heart's blood. Cultures of meningococci.

Guinea-pig 12.—Injected intraperitoneally with 4 c.c. of peritoneal exudate heated to 65° for one hour. No effect produced.

From the above experiments it is seen that four animals were inoculated successively with 0.5 c.c. of peritoneal exudate and all died in a short time of meningococcus septicemia with enormous numbers of meningococci in the peritoneal exudate. It is certain there must have been a rapid multiplication of the cocci in the animals. Furthermore, when heated sufficiently to kill the organism, the same or much larger quantities have little or no effect. There is a suggestion also of an increase of virulence, as shown by the result in Pig 2 and Pig 5. However, the experiment was not devised to show this and the result is not conclusive.

Injection of patients with killed meningococci.—Case 1.—Adult; typical case of epidemic cerebrospinal meningitis. Meningococcus was obtained from cerebrospinal fluid and nasal mucosa. Blood culture sterile. During the first few weeks the temperature varied from 100 to 104. It then became more irregular, varying from normal to 103. Patient became very emaciated. Leucocytic count at the end of the first week was 9,200; on the 11th day 10,500. During the sixth week of the disease while running the characteristic irregular temperature the patient was injected with heated organisms from the cultures obtained from the cerebrospinal fluid. The growth from several small blood-serum slants was suspended in 3 c.c. of salt solution, heated to 65° for 30 minutes and injected subcutaneously into the right arm. Six days later a similar injection was made into the left arm. No subjective symptoms of any importance occurred. Locally on the day after the first injection there was some tenderness and slight redness and swelling at the point of injection. This completely disappeared in four days. Following the second injection the local reaction was slightly more marked. A small abscess formed after a few days and 2 c.c. of sterile pus was withdrawn with a syringe. No meningococci were obtained in smear or culture. The leucocyte curve showed a prompt rise following each injection with a more gradual fall to normal in the course of about three days. The reaction was more marked following the second injection. The reaction, as manifested by the temperature curve, is hardly perceptible. Slight increase followed each injection but since the

[1] This amount of heating kills the organism.

temperature had been so variable previously, not much significance can be attributed to this rise. But it is to be noted that on the second day after the last injection the temperature fell to normal, and at no time subsequently did it rise above 98.8° F. The pulse which previously ranged from 90 to 150 gradually became more regular and soon became normal. The patient improved rapidly and in four weeks left the hospital in good condition and with no serious after-effects.

Case 11.—Adult; typical case; meningococcus obtained in pure culture from the cerebrospinal fluid. Blood cultures sterile. The temperature varied in a characteristic manner from 98 to 104. At times the patient had severe pains in the head and marked gastric disturbance manifested chiefly by severe nausea and vomiting. Opportunity was afforded to give one injection only. A 24 hours' growth was suspended in 3 c.c. of salt solution heated to 65° C. for 30 minutes and injected subcutaneously into the right arm. No subjective symptoms whatever occurred. On the following day there was a mild local reaction manifested by some tenderness, redness, and swelling. This completely disappeared in four days; no abscess formed. On the following day the temperature remained nearly normal and the leucocyte count was slightly lower. On the second day after the injection there occurred a slight rise in both the temperature and leucocyte curves. For about two weeks the patient remained in much the same condition with perhaps slight improvement. Later vomiting became more severe, she complained frequently of much pain in the head, and her general condition became worse. She died in the 10th week of the disease or five weeks after the vaccination.

The injection evidently had practically no effect.

Injection of heated meningococci into normal person.—The same organism heated in identically the same manner and in the same amount as used in Case 11 was injected subcutaneously in the left arm of the writer. The result was as follows: Immediately after the injection some smarting occurred locally and in a few moments became associated with a dull ache running down the arm into the hand. In 20 minutes nausea and vomiting took place. In 30 minutes occurred a severe chill lasting for half an hour, and shortly after, intense headache and muscular pain beginning in the calves of the legs and soon passing upward to all the muscles of the body. With this purging and vomiting of bile. In three hours the temperature rose to 103° F. During the remainder of the day and in the night the nausea and vomiting continued with headache, thirst, and marked prostration. On the following day there was some improvement. The nausea and vomiting subsided; temperature ran from 101 to 102° F. A diffuse rash appeared most apparent over the back; no petechia; the face was flushed; some stupor.

On the second day after the injection the rash disappeared. Slight improvement over condition on previous day. Temperature

between 99 and 100°. The urine contained large numbers of granular and a few hyaline and epithelial casts.

On the third day there developed an extensive herpetic eruption on the inner half of both left eyelids, on both lips, and on the mucous membrane inside of the mouth, chiefly over the hard palate. Tongue was heavily coated, breath offensive, and some nausea existed. Urine contained many granular casts.

On the fourth day marked improvement. Urine still contained a few casts and trace of albumin. From this time on gradual improvement occurred with no complications of any kind.

At the site of injection there was some redness, swelling, and tenderness for three or four days. No abscess formed and at no time were the axillary glands enlarged.

The temperature, leucocyte, and opsonic curves are given in Chart 1.

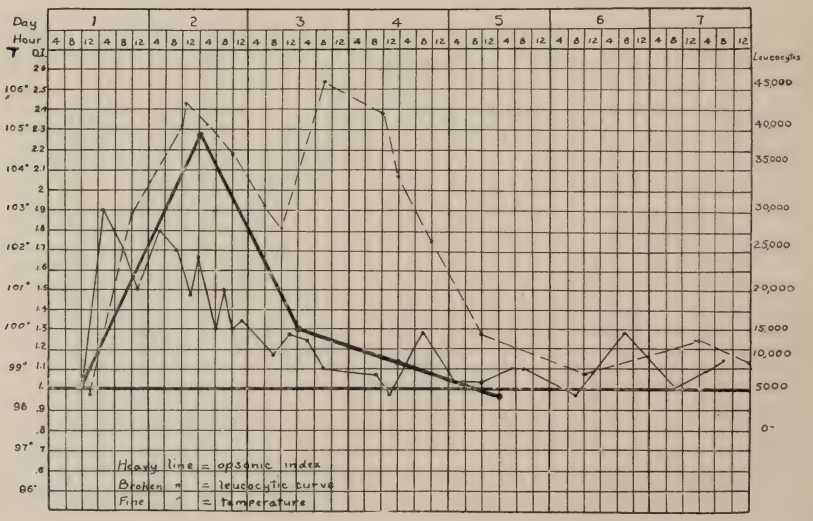

CHART 1.—Opsonic, Leucocytic, and Temperature Curves Following the Injection into a Normal Person of Heated Meningococci.

The temperature promptly rose to 103 and then followed a rather gradual decline, reaching normal on the morning of the fourth day. For nearly a month there was a slight evening temperature amounting to about one degree. With this there was some headache and slight indisposition.

The leucocyte curve also rose promptly. The maximum was reached on the third day when the white cells numbered 44,050. They rapidly disappeared and reached normal on the fifth day.

The opsonic index was determined each day. The organism was the same as that injected. Falling quantities of serum were employed, and in the preparation of the curve the sum total of all the cocci taken up in all the dilutions was used. A similar but not so uniform a curve may be obtained if instead of the sum total, any one of the dilutions is used. The character of this curve is very similar to the leucocyte curve. The rise is prompt and the decline is more gradual, the normal being reached on the fifth day. There was no negative phase unless it should have occurred between the time of the injection and the time the index was determined on the following day.

There was here a prompt and marked reaction in a normal individual from the subcutaneous injection of dead meningococci. The symptoms were those of a profound toxemia and no special meningeal symptoms occurred except those attributable to a general toxemia involving the central nervous system. The meningococcal substance is readily diffusible, as indicated by the very prompt reaction; this occurring in 20 minutes. It affected especially the nervous system because of the violent headache, slight delirium, and vomiting. Of interest is the extensive herpes, pointing most probably to an involvement of the Gasserian ganglia, which is in accord with the frequency of herpes in the disease as it occurs naturally. There was produced a marked acute nephritis. No focal symptoms occurred.

There was also a sudden reaction on the part of the protecting mechanisms of the body as shown by the temperature, leucocytic, and opsonic curves. The close correspondence in the rise and fall of the curves is very striking. The leucocytes increased in numbers about eight times and the opsonin more than doubled in amount according to the curve. The sum total of this increase would therefore be highly significant.

As already stated the organism and the amount injected in the normal individual were identical with that used in one of the meningitis cases. The results are strikingly different. The most probable explanation is that the individual with meningitis had acquired a marked immunity and the relatively large number of cocci had

almost no effect. When one considers the large amount of spinal fluid and the large number of meningococci often present not only in the fluid but in the blood and other parts of such patients, the number of organisms injected in these cases must be quite insignificant; consequently the effect in the patient need not be marked.

One must be extremely cautious in drawing any sweeping conclusions from such experiments. It is quite possible, indeed probable, that normal individuals vary in their susceptibility to the effect of meningococci and the same amount injected into another normal individual might produce possibly milder, possibly more severe symptoms. Pointing in this direction is the variation in the meningococcidal property of normal bloods already discussed. The variation of the virulence of the organism is another factor of great importance and must be considered in every instance. Again animal experiments must not be used as reliable indicators of the possible results upon human beings. What may have practically no effect upon a rabbit may cause profound toxemia in man, as was true in this instance.

The above data are entirely inadequate to make any definite statement concerning the value of killed meningococci in meningitis. The following suggestion may be made: In the early stages—during the first week—the organism has established very little if any immunity,[1] consequently only very minute amounts of killed cocci should be used at this time. From this time on increasing doses may be given watching the temperature, leucocyte and opsonic curves from day to day. The above curves suggest that injections might be made every 6 to 8 days. So many cases of this form of meningitis run a protracted subacute or chronic course, the temperature variations being very marked even for weeks, and often resulting in some irreparable deformity. Such cases, it would seem offer the greatest inducement for therapeutic inoculations.

NOTE.—Since this article was written several papers have appeared on the subject. Houston & Rankin (*Lancet*, 1907, 172, p. 1213) report upon the opsonic findings in 63 cases of cerebrospinal fever. From the sixth day on, all the cases showed an opsonic index of over 4.0; in some cases the index was very much higher. They call attention to its value in diagnosis. Von Eberts and Hill (*Am. Jour. Med. Sci.*, 1907, 134, p. 35) inoculated three cases of cerebrospinal fever with heated meningococci.

[1] The reaction of agglutination appears about the end of the first week.

Opsonic determinations showed a considerable increase following the inoculation. Two cases recovered and a third terminated fatally. Birnie and Smith (*Am. Jour. Med. Sci.*, 1907, 134, p. 582) report a case which showed an increase in the opsonic index following the injection of a meningococcus vaccine.

In the above cases treated with the vaccine the question arises as to how much the increase in the opsonin is due to the injection of the dead cocci and how much to the increase that naturally occurs in the course of the disease as shown by the observations of Houston and Rankin.

ON THE ROUTE OF ABSORPTION OF BACTERIA FROM THE PERITONEAL CAVITY.*

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It has been a generally accepted teaching that the chief route of absorption of the contents of the great serous cavities is furnished by the lymphatic system, and although the possibility has been sometimes admitted that soluble substances might to some extent diffuse into the blood vessels of the serosa, yet it seems to have been assumed that all formed particles must escape through the lymph channels. In recent years it has been shown by Asher, Starling, Mendel,[1] and others that absorption and removal of soluble substances introduced into the peritoneal cavity is accomplished through the blood vessels to a large extent, indeed, apparently to even a greater extent than through the lymphatics. In performing experiments which involved consideration of the route of absorption of a finely divided emulsion from the peritoneal cavity,[2] it was found that the emulsion did not appear in the lymph flowing from the thoracic duct in any appreciable quantities, and the question then presented itself: Is the assumption which is current among pathologists and surgeons, that bacteria leave the peritoneal cavity in peritonitis chiefly or solely by way of the lymph stream, a correct assumption? Are there any proofs that this is the route followed by the bacteria in entering the blood from the peritoneal cavity. Is it not probable that bacteria migrate directly into the blood vessels fully as readily as into the lymphatics?

Many *a priori* arguments in favor of the latter view presented themselves. For example, our views of absorption of formed particles by way of the lymphatics rather than by the blood stream are largely the outcome of the long-cherished belief that the lymphatic capillaries communicate directly with the serous cavities by means of stomata, and hence offer an open avenue of escape for bacteria and similar insoluble particles; yet there now seem to be ample grounds

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[1] *Amer. Jour. Physiol.*, 1899, 2, p. 342.

[2] Wells and Mendel, *Amer. Jour. Physiol.*, 1907, 18, p. 156.

for believing that the lymphatics are closed vessels, and if so they should be no more readily entered by bacteria than the blood capillaries. Another argument, also, is the accumulating evidence that bacteria injected into the peritoneum appear in the systemic blood stream in large numbers within a very few minutes, a rapidity quite at variance with the known slowness of the lymph flow. Furthermore the lymph glands should offer a decided barrier to rapid passage of bacteria into the blood, if they exercise the function commonly ascribed to them.

The literature contains several reports upon the question of the absorption of bacteria from the peritoneal cavity, but in none of them has it been definitely determined to what extent bacteria pass from the peritoneum to the blood by way of the lymphatic system. To be sure, several observations have been made bearing on this point in one way or another, but the positive proof obtainable by studying the bacterial content of the lymph flowing from the thoracic duct seems not to have been sought; at least no reference to such studies has been found in any of the more recent literature on the subject of peritoneal absorption.

The most important observations on the absorption of bacteria from the peritoneal cavity have been made by Buxton.[1] This investigator found that if large numbers of typhoid bacilli (one-half an agar slant) are injected into the peritoneal cavity of rabbits, large numbers of bacilli appear in the blood very quickly. The largest numbers are found in blood taken from five to fifteen minutes after the inoculation, although this large number generally persists in the blood for thirty minutes or so, and then decreases rapidly, so that as a rule after one hour few bacteria can be found in the blood. This "rush" of bacteria into the blood is considered by Buxton to occur by way of the lymphatics, which the bacteria enter through the diaphragm. In support of this view is advanced the fact that at a correspondingly early period after inoculation large quantities of bacteria may be found in the anterior mediastinal glands, into which converge the lymphatic vessels of the diaphragm on their way to the thoracic duct. It has been found that small foreign particles other than bacteria, such as granular pigments, follow the same route,

[1] *Jour. Med. Res.*, 1906, 15, pp. 1–89; 1907, 16, pp. 17–42, 251.

Muscatello having found that in dogs carmine particles injected into the peritoneum reach these lymph nodes in five to seven minutes. According to Buxton's observations there is little if any absorption of pigment granules by any part of the peritoneal surfaces besides the diaphragm and omentum, but in both these structures the absorption of particles is extremely rapid, and they soon appear in the anterior mediastinal glands. No more positive evidence than the above seems to have been obtained that the route of entry of bacteria into the blood is through the lymphatics, and there seems to have been no attempt made to determine whether bacteria do or do not pass from the peritoneum into the blood stream directly.

In view of the accumulating evidence that soluble substances, both in the peritoneum and elsewhere, are absorbed directly into the blood, it is pertinent to inquire if bacteria may not do likewise. There are a number of observations recorded by Buxton that might be interpreted as evidence in favor of the direct absorption of bacteria into the blood. First, the fact that the blood contains the largest number of bacteria a very few minutes after intraperitoneal inoculation is more readily understood if bacteria can pass directly into the blood vessels than if they must, before reaching the blood, be first absorbed into the diaphragmatic lymphatics, from there pass to the mediastinal nodes, be filtered through the tortuous sinuses of one or more of these, and from there pass through another set of lymphatic vessels to the thoracic duct, and thus reach the blood. Anyone who has observed the very slow dripping of the thoracic lymph from a cannula in the duct of even a large animal, will be inclined to question the probability that any considerable number of bacteria can make this journey and swarm into the blood in great numbers in the space of five minutes. Again, the large numbers of bacteria found in the lymph nodes might be reasonably interpreted, not as bacteria on their way to the blood, but rather as bacteria kept back from the blood by the lymph nodes, which are, supposedly, interposed as a barrier to prevent just such a rush of bacteria into the blood. In such a set of experiments by Buxton, in which relatively small numbers of bacteria (20,000 to 50,000), were injected, it was found that a considerable number can be recovered from the blood, anterior mediastinal lymph glands, and liver when these are removed ten to fifteen

minutes later. "Organs other than the liver contain so few bacteria after these minimal doses that they have been left out of account in making up the table."[1] Might this observation not be interpreted in favor of the view that bacteria not only enter the lymph stream from the peritoneum, but also enter the portal vessels directly? If the bacteria entered the blood through the thoracic duct only, we should expect them to be at least as abundant in the other viscera as in the liver, which receives but little blood that has not already been filtered through two separate sets of capillaries. But if bacteria in the peritoneum can pass into both the blood and lymph directly, we should expect to find them arrested chiefly in the first two filters of these vessels, the liver and the anterior mediastinal glands, and this is exactly what Buxton has found to be the case. In Buxton's tables in the same article[2] it will be obserevd that there are about as many bacteria recovered from the anterior mediastinal glands as are present in the entire liver or in the entire blood of the animal. This might be interpreted as indicating either that absorption from the peritoneum by the lymphatics is about as great as by the blood, assuming that the lymph glands and the liver are equally effective as filters; or, in view of the very great bactericidal power of the blood as shown in these and other experiments, it is quite possible that even more bacteria are absorbed by the blood than by the lymph, since only part of the bacteria absorbed into the blood remain in a viable condition so that they can produce colonies when inoculated into plates.

In a later article[3] it is stated that at first there is a greater deposition of bacteria in the liver than in the spleen, but after four to six hours the number in the spleen increases until this organ contains more than the liver, and their eventual disappearance from the spleen is slower than from the liver. Is it not possible to interpret this observation as indicating that at first bacteria reach the blood chiefly by direct absorption into the portal capillaries, so that they are first found most abundantly in the liver; later, when they have slowly made their way through the lymphatics to the thoracic duct, they enter the general circulation, and the spleen exercises its highly developed phagocytic function to remove them from the blood?

[1] BUXTON, *Jour. Med. Res.*, 1907, 16, p. 25.

[2] *Loc. cit.*, pp. 28–31.

[3] *Jour. Med. Res.*, 1907, 16, p. 254.

As can be seen, we find in the results of Buxton's careful experiments no conclusive evidence in favor of the prevailing assumption that bacteria are absorbed from the peritoneal cavity either chiefly or exclusively by the way of the lymphatics; but rather, find excellent reasons for believing that bacteria may enter the blood vessels directly as well as the lymph vessels, exactly as has been found in studies of the absorption of soluble substances.

The only definite consideration of the pros and cons of the question of the direct absorption of bacteria into the blood that we have found, is contained in the recent article by W. Noetzel.[1] This author quotes the observations of Schimmelbusch, that bacteria may be absorbed from fresh wounds directly into the blood, where they may be found in ten minutes. Noetzel found that anthrax bacilli injected into the peritoneal cavity of animals appear in the circulating blood in ten minutes. *Bacillus pyocyaneus* injected into the pleura was found in the blood in an equally brief space of time. He discusses the question of the possibility of the rate of the lymphatic flow being great enough to account for so rapid a transportation of bacteria from the serous cavities into the blood, and performed experiments that seem to answer it affirmatively. These experiments were as follows: Bacteria (*B. pyocyaneus*) were injected into the knee joints of rabbits care being taken to avoid any injury to blood vessels, apparently with the idea in mind that bacteria can enter the blood vessels only when these have been injured. The bacteria were found in the blood stream as early as five minutes after such injection, and Noetzel believes them to have been transported solely by the lymphatics, since he had injured no blood vessels. Of more importance is his observation that the inguinal, crural, and lumbar lymph glands, removed five or ten minutes after injection of the bacteria into the knee joint, contained the same variety of organism; the numbers were not ascertained. This seems to be good evidence that absorption of bacteria from the synovial sacs takes place in a very few minutes, and that bacteria may pass in this time beyond the first sets of interposed lymph nodes. Dismissing the possibility that bacteria can enter the uninjured blood vessels of the synovial sacs directly, without having disproved it, Noetzel considers that all the bacteria

[1] *Beitr. z. klin. Chir.*, 1906, 51, p. 740.

found in the blood must have passed through the lymphatic system from the knee joint to the thoracic duct; to account for this he introduces evidence that the lymph stream may escape passing through the lymph nodes, because of the presence of anastomosing branches of the lymph vessels in the capsule of the lymph nodes which connect the afferent and efferent lymph vessels, and also through branches connecting the larger trunks directly with one another. As a result of his studies and deductions he is very sceptical as to the value of the lymph nodes as filters of the lymph, and doubts that they have the protective function against bacterial invasion so universally ascribed to them.

Other writers on the subject of the absorption of bacteria seem entirely to have neglected consideration of the route by which the absorption takes place, or else they have assumed that the lymphatics offer the only possible route. Apparently they have all been influenced by the current view that the lymphatics open directly into the serous cavities, and hence offer a free avenue of escape to the bacteria, while believing, as does Noetzel, that the only formed substances that can possibly pass through the walls of the blood capillaries are the leucocytes. If the lymph capillaries do not have stomata opening directly into the serous cavities, as seems to have been established definitely by histological and embryological studies, then there is no evident reason why bacteria may not enter the blood capillaries exactly as readily as they enter the lymph capillaries. However, it is not to be denied that there are many features of absorption, both of solid particles and of dissolved substances, that are much more difficult to explain if there are no stomata than if the presence of stomata is assumed, and at the present time it would seem unwarranted to refuse to admit the possibility that there may be functional stomata, even although they are not anatomically demonstrable.

With the above facts and hypotheses in mind, we have attempted to ascertain by direct experiment: First, whether bacteria injected into the peritoneal cavity do or do not find their way into the thoracic duct, and if so, after what length of time they reach the subclavian vein. Second, whether they enter the blood without passing through the thoracic duct. The experiments have given us very conclusive

results as far as they have been carried out, and therefore it seems desirable to publish them although there are many features of the problem still left undecided, since we are unable to continue the work at this time.

The experiments were performed as follows: Dogs were anesthetized with A.C.E. mixture, after a preliminary dose of morphine. A sterile paraffined cannula was inserted into the femoral artery and another into the main trunk of the thoracic duct just before it enters into the vein, the fluid from the latter dripping into a sterile graduated cylinder. An emulsion containing the washings from several agar slants covered with a 24-hour growth of bacteria in 0.85 per cent salt solution was injected by means of a blunt pipette into the peritoneal cavity through an incised opening, and the peritoneal wound closed by either a purse-string suture or by artery forceps in order to bring peritoneal surfaces in contact. Blood was drawn off at intervals from the cannula in the femoral artery into sterile test tubes, always first allowing enough blood to escape to ensure getting blood from the general circulation; 0.5 c.c. was then measured off in a sterile pipette, mixed with melted agar and plated out before coagulation occurred. We had no difficulty in securing cultures in this way without having the blood clot, and did not find it necessary to use bile or other coagulation-inhibiting substances. At similar intervals two drops of the lymph were allowed to fall into a tube of melted agar, and plated out. When streptococci were being used in the experiments the lymph was drawn into freshly made blood agar, in order to increase the growth and to facilitate the recognition and counting of the colonies. With rabbits the procedure was the same, except that we were unable to insert a cannula into the thoracic duct, therefore the duct when found was divided, and when not found all the tissues where the duct lies, and also the great veins in the vicinity, were securely occluded by forceps to shut off the lymph from the blood. Hence in the rabbits we did not make cultures of the thoracic lymph itself, but determined how many bacteria were present in the blood of animals with this channel shut off, and how many were in the blood of normal rabbits of the same size treated in the same way except that the thoracic duct was untouched.

The results of the experiments were as follows:

Experiment II.[1]—A large male hound, weighing about 25 kilos, was treated as above described. One hundred and twenty-five c.c. of salt solution containing the growth on 10 slants of agar during 24 hours, were injected into the peritoneal cavity. From the cannula inserted in the thoracic duct lymph flowed at the rate of 6 c.c. in the first 10 minutes, and 3 c.c. in 10 minutes in the middle of the experiment, which is about the normal rate for dogs of this size. The cannula was not paraffined in this experiment, and became occluded frequently by clots, which had to be removed with a sterile platinum wire; therefore there occured considerable contamination of some of the plates, but in the figures given below all but the colon colonies are disregarded.

TABLE 1.

COLON COLONIES FROM LYMPH FROM THORACIC DUCT (2 DROPS USED FOR EACH PLATE).

Time after Injection	Number of Colonies
2 min.	0
7 "	0
14 "	0
21 "	0
32 "	87
45 "	210
53 "	132
66 "	500
89 "	10
108 "	41
118 "	18

Cultures made from the femoral blood during the same period failed in every case to show colonies of colon bacilli.

This experiment furnishes evidence that colon bacilli when, injected in great numbers into the peritoneum of dogs, enter the thoracic duct and begin to reach the thoracic duct blood in about half an hour; after the end of the first hour the number rapidly decreases. In the same animal no living bacilli were present in the blood drawn from the femoral artery. A similar result was obtained in the following experiment:

Experiment III.—Male dog, weighing about 10 kilos, treated in the usual way. Besides the main thoracic duct, two small branches were found entering the vein; these were ligated and a paraffined cannula inserted into the main trunk. The lymph flow was always good, the rate being 10 c.c. in the first five minutes, and from 5 to 7 c.c. in 10 minutes during the middle of the experiment. One hundred cubic centimeters of salt solution containing 24 hours' growth of colon bacilli on seven agar slants were injected into the peritoneal cavity, and the results of the culures were as follows:

[1] The first experiment was of doubtful value because of faulty technique, the cannula in the thoracic duct becoming frequently occluded by clots, so that no lymph could be obtained. Cultures from the blood made during two hours did not show any colon colonies.

TABLE 2.

Time after Injection	Colonies from Lymph (2 Drops)
5 min.	0
12 "	10
18 "	53
25 "	81
32 "	279
41 "	311
48 "	188
57 "	180
69 "	45
81 "	14
99 "	10
111 "	6
119 "	3

In none of the cultures from the blood of the femoral artery were colon colonies found.

Here again we find a large number of colon bacilli reaching the end of the thoracic duct in a half-hour, the flow being somewhat more rapid than in the previous experiment in which the cannula became frequently occluded by clots, with the number decreasing rapidly in an hour. Likewise no colon bacilli could be found in the arterial blood during the two hours the experiment was continued. These results would seem to indicate that bacteria escaping from the peritoneal cavity of dogs reach the blood only through the thoracic duct, and do not pass directly into the blood. To control this, however, it was necessary to ascertain the number of bacteria reaching the femoral blood of dogs with intact lymphatic vessels, as in the following experiment:

Experiment IV.—Male dog, weighing about 7 kilos, anesthetized in the usual way, and injected intraperitoneally with 100 c.c. of salt solution containing 10 slants of colon culture. The neck region was left untouched, and plate cultures of the blood from the femoral artery were made at frequent intervals during two hours. *In not a single plate did colon colonies appear.*

Although the thoracic duct of this dog was intact, and living bacteria were passing into the blood in the lymph at a rate of several hundreds or thousands a minute, if we can judge by the results of other experiments, yet no bacteria could be found in the blood escaping from the femoral artery. Evidently the power of the blood of the dog to kill colon bacilli as used in this experiment is great enough to destroy all those reaching it from the peritoneum, either through the lymph or directly, and consequently our first two experiments prove only that bacteria pass from the peritoneum into the lymphatics; they do not prove that bacteria are not absorbed directly

into the blood from the peritoneal cavity. In the hope that we might be able to learn something about this possibility a similar set of experiments was performed with a strain of virulent streptococci, which it was thought might be able to survive the bactericidal power of the dog's blood long enough to be detected in the arterial blood. These experiments resulted as follows:

Experiment V.—Brindled female, very fat, weighing about 15 kilos, was prepared in the usual way. The thoracic duct was found and ligated, but having difficulty because of the very great obesity of the animal, did not succeed in inserting a cannula. Injected into the peritoneal cavity 100 c.c. of salt solution containing streptococci from five blood-agar slants of streptococci, grown 24 hours.

TABLE 3.

Time after Injection	Colonies from Blood (0.5 c.c.)
2 min.	0
5 "	0
15 "	0
45 "	0
60 "	3
75 "	0
90 "	4
105 "	1

Experiment X.—Small male dog, weight 5 kilos, anesthetized as usual. Cannula inserted in femoral artery; nothing done to thoracic duct. Injected 100 c.c. of salt solution containing five slants of streptococci grown 48 hours.

Made cultures at intervals for an hour from the blood, but obtained *no* streptococcus colonies.

Experiment XIII.—Large male dog, weighing about 20 kilos. Cannula inserted in thoracic duct, and obtained a good flow of lymph; 7 c.c. in the first ten minutes, and the same rate at the end of one hour. Injected into the peritoneum 100 c.c. of salt solution containing 10 slants of streptococcus, 48 hours old. The results of the cultures were as follows:

TABLE 4.

Time after Injection	Colonies from Lymph (2 Drops)	Colonies from Blood (0.5 c.c.)
2 min.	0	..
7 "	0	0
15 "	2	7
20 "	15	..
25 "	....	0
30 "	100*	..
35 "	1,000	0
40 "	2,000	..
45 "	50	0
60 "	30	1
75 "	40	0
90 "	...	0
105 "	...	0
120 "	...	0

* Numbers approximated in this column.

These experiments show that streptococci, like colon bacilli, reach the thoracic duct in considerable numbers after about half an

hour, but the number present in the lymph soon decreases about as rapidly as it begins. We also find evidence that streptococci may possibly get from the peritoneum into the blood by some other route than the thoracic duct, since a few bacteria were found in the blood in Experiments V and XIII, although it is possible that they may simply have passed from some anastomosing branch of the thoracic duct to the lymphatics entering the veins on the right side of the neck. As the control animal, No. X, with the thoracic duct intact, showed no cocci in the blood from the femoral artery, it is impossible to tell whether the small number of cocci found in the blood of the other two animals indicates that the number of bacteria reaching the blood by some route other than the thoracic duct is small, since it is evident that such bacteria would soon be destroyed through the bactericidal power of the blood. It seems that the latter factor is so great in dogs that they are unsuitable for determining this point, and hence experiments were performed with rabbits. In rabbits the thoracic duct is so small and delicate that we were unable to insert a cannula, but contented ourselves with occluding it and comparing the bacterial content of the blood of such animals with those in which the thoracic duct was untouched. Apparently the bactericidal power of the blood of rabbits for colon bacilli is not so great as in dogs, for there was no difficulty in finding great numbers of bacteria in the blood soon after they had been injected into the peritoneum, as shown by the following experiments:

Experiments VI and VII.—Two rabbits, three-fourths grown, of about equal size. Into the peritoneal cavity of each were injected 25 c.c. of salt solution containing one-half the growth of colon bacilli on an agar slant of 24 hours. With No. VI, cultures were made at intervals from the femoral artery. With No. VII, the thoracic duct was first isolated and cut, so that no lymph reached the blood from this source. The results of the blood cultures in each case were as given below:

TABLE 5.

Time after Injection	VI (Control) Colonies from blood (0.5 c.c.)	VII (Duct Cut). Colonies from Blood (0.5 c.c.)
1 min.	0	0
3 "	1	0
5 "	272	0
10 "	66	0
15 "	75	12
20 "	21	2
25 "	22	0
30 "	5	0
35 "	2	0
40 "	?	0
50 "	6	0
60 "	3	0

Experiments VIII and IX.—In these experiments the conditions were the same as in the previous pair, except that, being unable to find the thoracic duct, it was occluded by artery forceps grasping the tissues where it lies, while other forceps shut off from the general circulation that part of the jugular and cervical veins into which the lymph could possibly be discharged. The results were as follows:

TABLE 6.

Time after Infection	IX (Control). Colonies from Blood (0.5 c.c.)	VIII (Duct Occluded). Colonies from Blood (0.5 c.c.)
5 min.	0	0
10 "	0	0
15 "	560	0
25 "	1,270	0
35 "	460	0
45 "	590	0

TABLE 7.

EXPERIMENTS XI AND XII. CONDITIONS THE SAME AS ABOVE.

Time after Injection	XI (Control). Colonies from Blood (0.5 c.c.)	XII (Duct Occluded). Colonies from Blood (0.5 c.c.)
5 min.	240*	44
10 "	240	66
15 "	350	..
25 "	Spoiled	Lost
35 "	2,000	87
50 "	3,000	21
60 "	2,000	0

* Numbers approximated in this column.

These experiments seem to show conclusively that when colon bacilli are injected in large numbers into the peritoneal cavity of rabbits, they pass into the blood chiefly by way of the thoracic duct, for when the duct is cut or occluded relatively few or no bacteria can be found in the arterial blood. The bacteria reach the blood in a very few minutes, and either decrease rapidly after the first "rush" of bacteria, or else they may continue to increase (Exp. XI), presumably because of deficient defensive powers of the blood. Since in two of these three experiments a greater or less number of colon bacilli were found in the blood in spite of the occlusion of the thoracic duct, it seems probable that they can enter the blood by some other route. It must be emphasized, however, that as in experiments such as these in which the thoracic duct is occluded a stasis of the lymph current is produced, so that the conditions of absorption are not normal. It is quite possible that such occlusion of the main duct causes the lymph to reach the blood by collateral branches

passing to the lymph duct on the right side, or possibly by some other less understood route. Therefore the finding of bacteria in the blood of rabbits with occluded thoracic ducts cannot be considered positive proof that the bacteria have entered the blood directly rather than by way of the lymphatic vessels. It would be very interesting to study the bacterial content of the portal blood of animals receiving intraperitoneal injections of bacteria, but we have been unable to devise a method of doing this in the living animal without inflicting severe peritoneal trauma and making the conditions of absorption altogether abnormal.

SUMMARY.

Evidence has been secured that when colon bacilli or streptococci are injected in large numbers into the peritoneal cavity of dogs or rabbits, they begin to enter the blood through the thoracic duct in considerable numbers after from 15 to 30 minutes in the dog, and from 5 to 15 minutes in the rabbit. The difference in the length of time probably depends upon the distance the organisms have to travel in the slowly moving lymph. It is possible that some bacteria enter the blood by some other route than the thoracic duct, but whether the route consists of collateral lymph channels, or direct passage into the peritoneal blood capillaries, we have not ascertained; neither have we been able to determine positively whether bacteria do or do not escape directly from the peritoneum into the blood. There is a striking rush of bacteria into the thoracic lymph, so that the maximum number is found almost at once after the first bacteria appear, and begins to decrease in half an hour or less; this corresponds with the observations of Buxton upon the appearance of bacteria in the circulating blood after their injection into the peritoneum.

It would seem probable that the mechanism of absorption of bacteria from the peritoneal cavity is quite different from the mechanism of absorption of soluble substances, since the latter seem to pass directly into the blood even more rapidly and abundantly than into the lymph. This is comparable to the condition of absorption in the intestine, the soluble sugar entering the blood directly by diffusion while the emulsionized fat escapes through the lymphatics.

THE EFFECT OF THE INJECTION OF KILLED STREPTOCOCCI ON THE STREPTOCOCCO-OPSONIC INDEX OF NORMAL RABBITS.*

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With the hope of arriving at a better understanding of the effects of the injection of killed bacteria on the opsonic index in cases in infection, it was thought wise to determine the effect of such injections on the opsonic index in normal animals. A so-called vaccine was accordingly prepared by Dr. Weaver from a streptococcus isolated from the tonsil of a patient with scarlet fever. Twenty-hour growths on ascites-agar were suspended in NaCl solution and heated to 65° C. for two hours. Before heating, smears showed only streptococci; and after heating cultures made from the suspension remained sterile. Immediately before heating it was estimated by Wright's method and by plates that each c.mm. of suspension contained 350 millions streptococci. Dilutions of the suspension were used in the experiments to be described.

Normal rabbits were injected and with three chief objects in view: (1) To determine the effect on the opsonic index of different quantities of killed streptococci injected at intervals of several days or after such cycle of events as might follow each injection had been completed; (2) to observe any differences in results produced by intravenous and subcutaneous injections; (3) to determine the effect of very frequent or daily injections on the opsonic index with especial reference to possibility of producing a persistent negative phase.

As a preliminary to making these experiments the normal streptococco-opsonic index of the rabbits to be used was determined every second day, five estimations being made for each animal. The sera of three other normal rabbits were used as a pool. An average of 15 estimations gave an index of 1.05. The indices varied as a rule between 0.80 and 1.20, the lowest was 0.76, the highest 1.40. This high index was observed in normal rabbits but once, 1.27 being next highest.

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The technique used was that generally employed in estimations of opsonic indices. The pooled serum consisted of equal parts of sera from three normal rabbits. Both human and rabbit washed leucocytes were used as phagocytes at first, but the results being practically the same in both cases, during the remainder of the experiments, only human leucocytes were used. The results given are those obtained with human leucocytes.

The experiments directed toward the determination of the effect of intravenous and subcutaneous injections of dead streptococci

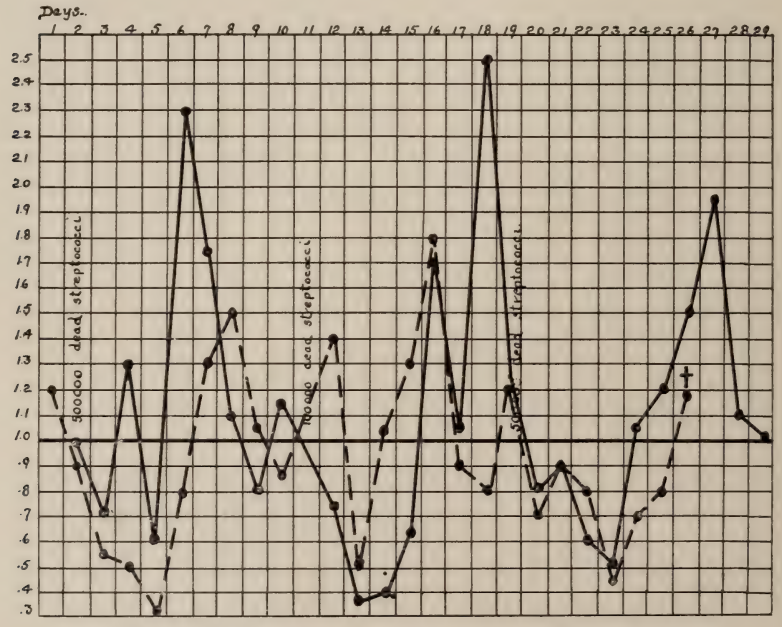

Chart 1.—Streptococco-opsonic Index of Rabbits Injected with Dead Streptococci.

Solid Line=subcutaneous injection.
Broken " =intravenous "

upon the opsonic index to the homologous streptococcus extended over a period of 30 days. Charts 1 and 2 show the dates of injections, the amount injected and the time of taking the blood for estimation of the opsonic index. Each animal charted received three injections. Seven days after receiving the third dose of vaccine, one of the rabbits (III) died (Chart 1). At autopsy no gross changes could be detected

and cultures from the heart's blood and bile remained sterile. It is to be noted, however, that the streptococco-opsonic index had been below 1.00 for five days. No other perceptible deleterious effects from the use of the vaccines could be determined. The temperature of the animals, taken for several days after the first injection, showed some fluctuation but did not vary much above or below the maximum and minimum temperatures of six normal rabbits. There was no significant loss of weight.

The results of these experiments seem to indicate, (1) that small doses subcutaneously produce the greatest rise in the opsonic index

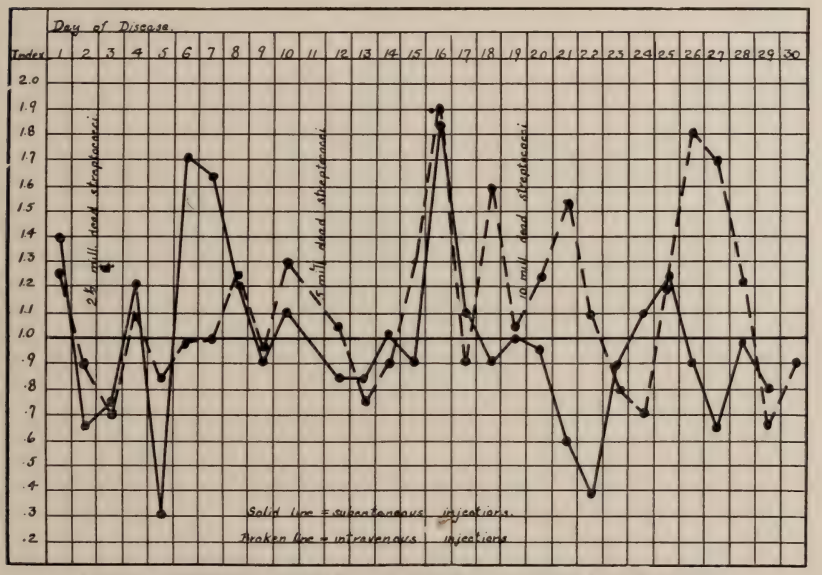

CHART 2.—Streptococco-opsonic Index of Rabbits Injected with Dead Streptococci.

(Chart 1). In these experiments the highest index, 2.50, was obtained after injection of 100,000 dead streptococci subcutaneously. As each succeeding dose was double the preceding one, it is to be noted that the best results really were obtained with the first and smallest dose. After the second injection it is true that the index reached a point 0.13 higher than after the first injection, but it remained above normal only two days and the rise was preceded by a much more marked depression than the first injection. (2) That as a rule there is a definite cycle of events after each injection, a "negative phase," a

"positive phase," and a return to normal. In several instances there occurred in these experiments a preliminary rise followed by a fall in the index, and later by a more marked rise, i. e., the positive phase proper; this is especially well shown in Chart 1. A curious feature is illustrated in Charts 1 and 2, namely, that after the second and third injections a longer time was required for the index to reach the maximum than after the preceding injection. Thus in Chart 1, the highest point after subcutaneous injection was reached four, seven, and eight days after the first, second, and third injections respectively. The cycle of events after intravenous injections is more irregular and atypical than after the subcutaneous.

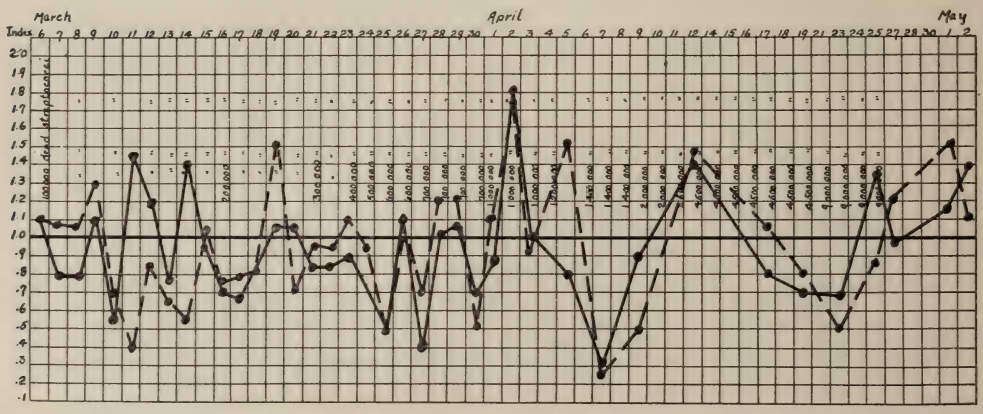

Chart 3.—Streptococro-opsonic Index of Two Rabbits Receiving Daily Injection of Dead Streptococci.

The experiments to determine the effect on the opsonic index of very frequent and increasing doses of dead streptococci were made along much the same lines as the preceding and were continued over a period of 56 days (Chart 3). Two rabbits were injected, at first every second day, later every day, with increasing doses of dead streptococci. To begin with, doses containing 50,000 dead streptococci were used, the doses being gradually increased until 9,000,000 dead streptococci were given subcutaneously each day. An effort was made to inject each time into the same place. The opsonic index was estimated daily at first, later at less frequent intervals.

For the first 20 days there was a slow, but steady loss of weight by both animals, one losing 180 grams, the other 230 grams. During the last 37 days of the experiment both rabbits regained much of their lost weight without quite reaching the standard shown before the injections were begun. The loss of weight could hardly have been due to the diet. During about the first two weeks of the experiment the animals were fed on carrots and oats, and on this feed both lost in weight. After this time only oats were fed and on this diet both rabbits at first lost, and later gained in weight. It seems likely, therefore, that the loss of weight was due to the effect of vaccine.

Chart 3 shows the effect of these injections on the opsonic index. These curves seem to indicate that, while daily injections of even large doses of dead streptococci do not keep the opsonic index permanently below normal, they do appear to have a restraining effect so that the index in these experiments was below the minimum normal limits a greater proportion of the time than in the experiments illustrated in Charts 1 and 2; nor did the index rise as high above normal nor remain up for so long a time as in these experiments. There seems to be a distinct tendency for the index to rise above normal after the injections have been continued for some time.

Five days after the last dose was given, one of these rabbits was killed because it was developing "snuffles." Post-mortem showed nothing abnormal except some scar tissue at the site of the numerous injections. On the same day the other rabbit was given a dose of live avirulent streptococci from two 24-hour agar slants. Single 24-hour agar slant growths of the same streptococcus did not kill normal rabbits. The double dose appeared to make the injected animal slightly sick but did not kill it. It would seem, therefore, that the resisting power of this rabbit was either not (markedly) reduced below normal or that the normal resistance was very quickly regained after discontinuance of the injections.

From these experiments the following conclusions seem permissible:

1. That single injections of 50,000 to 2,500,000 or more of dead streptococci in normal animals cause first a fall and later a marked rise in the streptococco-opsonic index.

2. That relatively small doses may cause greater rise in the index than larger doses, and that subcutaneous injections may be followed by more pronounced rise in the index than intravenous.

3. That daily injections of increasing doses of dead streptococci do not produce a permanent subnormal opsonic index in normal rabbits, but do have a distinctly restraining influence on the index as compared with the effect of single injections.

SOME OBSERVATIONS UPON OPSONINS IN PNEUMONIA AND IN FOUR STREPTOCOCCUS INFECTIONS.*

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METHOD.

THE technique was essentially that of Wright and Douglas. The bacterial emulsion was at first prepared by mixing the growth from agar in NaCl solution, and for the observations upon streptococci this method was adhered to on account of the necessity for thoroughly breaking up the chains; but for pneumococci, a 24-hour broth culture was found to furnish an emulsion of just the right thickness. A meat-extract broth, reaction + 1 per cent, gave constant results.

The smears were made with cigarette paper. This gave an even distribution (mainly along the edge), and caused little trauma to the leucocytes. In staining, trials were made to find a method which would stain the leucocyte so that its margins were sharp as shown by granulations, yet which would stain the organisms deeper than the nucleus, so that they could be readily seen when near or overlying the latter. For pus cocci we found that due to their great affinity for methylene blue, a few seconds' staining in Jenner gave excellent results. Pneumococci, however, were too faintly stained. When the slide was flooded with J. Homer Wright's stain for one to two minutes, rinsed, and counterstained with methylene azure for 10 to 20 seconds, the nucleus was reddish and the cocci deep purple.

About 25 sera were tried, using the usual Wright-Douglas method with the above additions. The sera were normal and from pneumonia patients; usually three samples were taken from each person. The source was unknown until after the estimations were made. Duplicate preparations were also made from one tube. The time of incubation was varied, 10 minutes giving the best results for pneumococci. With a longer time the cocci stained less sharply and

* Received for publication June 15, 1907.

intensely and were therefore difficult to count. The degree of accuracy was not very satisfactory; occasional figures showed 33 per cent or more difference, many 18 to 22 per cent, though in more than half the difference was much smaller. Counting 200 cells corrected such differences but slightly. It was noticed that the laboratory temperature made a decided difference in the degree of phagocytosis which was much lower on cold days. Warmed solutions were then employed. The pipettes were then calibrated so that equal quantities were used through one series. A system of counting the doubtful cells and cocci was then strictly adhered to, one point being to ignore packed leucocytes as accidental, another only to count isolated leucocytes, avoiding even the smaller clumps. Thorough mixing of the cream prevented most of such clumping. Adherence to these details diminished the variations materially. At least 10 per cent variation in the opsonic index may be due to the sources of error in the method and cannot be considered. Often the error may be more. The variations in using two sets of leucocytes are given later.

LOBAR PNEUMONIA.

Three fatal cases.—E. H., died of acute cardiac dilatation on 7th day. On 6th day, good condition, lower left lobe consolidated. Leucocytes 21,000. Opsonic index 0.96.

J. S., died with toxemia and hyperpyrexia. Just before death, leucocytes 11,000; polynuclears 73 per cent. Opsonic index 1.16.

J. M., spreading pneumonia, death 15th day, edema of lungs. On 10th day, leucocytes 15,000, polynuclears 88.5 per cent. Opsonic index 0.95. On 14th day, leucocytes 13,600, polynuclears 88 per cent. Opsonic index 0.89.

Four cases with crisis.—The opsonic index rose on the day of crisis, was highest the day after crisis, and fell within a few days to normal, the leucocytes and polynuclears falling. One case showed the following: On the sixth day in hospital a pseudo-crisis occurred, leucocytes 8,500, polynuclears 81.5 per cent. Opsonic index, 1.63. The temperature again rose, a second critical fall on 8th day, leucocytes 11,800, polynuclears 68 per cent. Opsonic index, 1.53.

Three cases with lysis.—One case showed a moderate rise during lysis to 1.29. A mild case showed no change. The third case already convalescent showed no change.

Two cases terminating with empyema.—One showed a beginning rise when resolution took place. The opsonic index was 1.50, 10 days later. One week later when pus was forming, it was still 1.33. Another case showed no rise.

BRONCHO-PNEUMONIA.

Isolated observations on two cases during fall of temperature showed a slight rise, to 1.25 in one case.

All opsonic indices taken during the course of the disease (except the one with pseudo-crisis) were approximately normal or slightly below.

STREPTOCOCCUS INFECTIONS.

Case 1.—J. M. B. Male. 30. Dr. S. W. Lambert. Entered New York Hospital October 22, 1906. Septicemia, ulcerative endocarditis; double pleurisy with effusion. Blood cultures gave *Strept. tenuis*. Leucocytes 10,000 to 12,000. Septic temperature 99° to 103°. Opsonic index to streptococcus from blood October 31, 0.46. Died November 2, before vaccine was completed. Not inoculated.

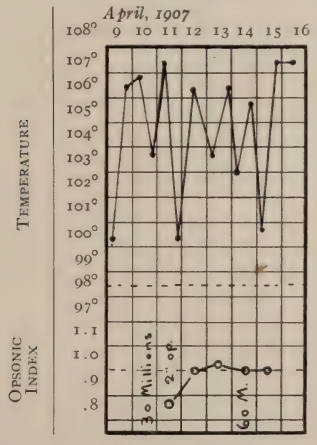
Case 2.—M. V. Female. 23. Seen with Dr. W. S. Bryant, April 10, 1907, at French Hospital. History of cold for seven days, earache for three days. Right mastoid operation April 5. Blood culture: streptococcus (by Dr. Krumwiede). Extensive ostitis. Very little pus. April 6, wound sloughed. April 7 and 8, dressed with horse serum, sloughing stopped. April 10, inoculated with 30 millions streptococci (A. R.), Case 3 below. April 11, second operation, excision of right internal jugular, curettage of lateral sinus. April 15, inoculated with 60 millions streptococci (A. R.). Became delirious. Metastases in left shoulder and right wrist. April 16, died. Autopsy showed meningitis.

Result of inoculation: no appreciable effect upon clinical condition nor upon opsonic index which remained normal. (See chart.)

CHART 1.

M. V. STREPTOCOCCUS SEPTICEMIA, MENINGITIS.

Inoculated with streptococcus from A. R.



Case 3.—A. R. Male. 20. Entered French Hospital February 15, 1907. No antecedent sore throat, venereal, or other illness. Four or five days before admission, pain in left shoulder, indigestion, vomiting, diarrhea. Three days before, pain in right knee, fever. Two days before, slight nose bleed and chilly feelings.

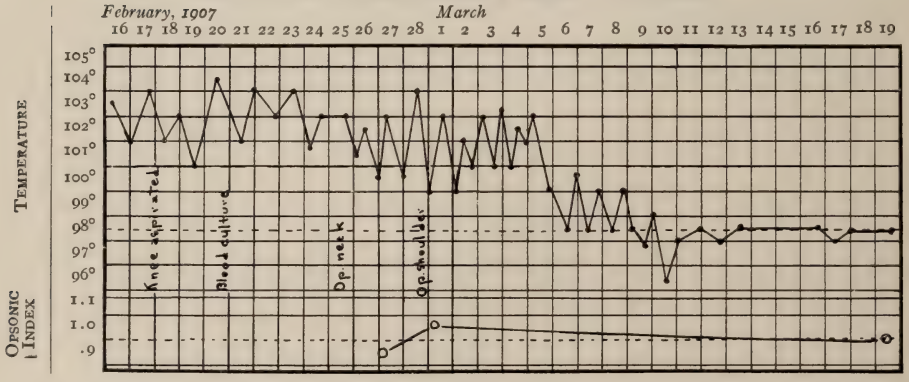
Physical examination: Cyanotic, flushed cheeks, eyes bright. Injected conjunctivae. Lips dry, heavily coated tongue. Swelling with edema, above and to the inside of the left shoulder joint, and over lower part of neck. Left arm held immobile. Attempt at motion apparently very painful, resisted by patient. Right knee enlarged,

tender; fluid in joint. Abdomen tympanitic. Spleen palpable, just below costal margin, soft. Liver, slightly enlarged, edge palpable. Nothing abnormal in rectum. No discharge from urethra. February 17, slight epitaxis. February 18, tongue dry, thickly coated, yellowish brown. Glands enlarged behind left sternocleidomastoid. Marked induration about shoulder, some little redness, heat, the edema has extended behind anterior edge of trapezius and to margin of sternum. Knee aspirated, 5 c.c. cloudy fluid: *Strept. pyogenes*. Delirious in evening, restless sleep. Liquids. February 19, faint soft systolic murmur heard at base and apex. Induration increased beneath left sternocleidomastoid. Right femoral and inguinal glands enlarged; rather soft. Femoral tender. Urine: amber, slightly cloudy, albumen trace, no sugar, few epithelial cells and leucocytes. February 20, slight conjunctival icterus. Edema about shoulder has extended slightly and is more brawny. Tongue cleaner. Patient rather somnolent. Catheterized. February 21, less somnolent. Pulse bounding. Urine: gave faint trace

CHART 2.

A. R. STREPTOCOCCUS SEPTICEMIA.

Synovitis of knee, abscess of neck and of shoulder. Not inoculated.



of albumen. Sediment as above. Blood culture: *Strept. pyogenes* (Dr. Krumwiede). February 22, slight nose bleed. Has slept better than any night. Much less restless. February 24, attempt was made to determine patient's opsonic index to streptococcus from his blood. No phagocytosis occurred. February 25, profuse perspiration. Vomited yellow curds. February 26, softening and localization behind sternocleidomastoid. Abscess opened under cocaine by Dr. Peck. Few drops of yellow pus, drained. Culture: *Strept. pyogenes*. February 27, several wheals on trunk. February 28, second attempt to determine patient's opsonic index successful. March 1, free perspiration at night. Urine: dark amber, few granular casts. March 2, Abscess about shoulder opened. Culture: *Strept. pyogenes*. March 3, patient's general condition continues the same. Involuntary micturition. Soft diet. March 5, vaccine prepared but its use delayed because patient's general condition had slightly improved. March 6, urine: no casts. March 9, wheals have gradually disappeared. Knee has gradually diminished in size, very slightly tender. March 13, urine: no casts. March 20, urine; 1,016, albumen none, no casts. March 27, opening in shoulder almost healed. April 2, wound completely healed. Patient was discharged cured. Except for the difficulty in determining the opsonic index and for a delay in the preparation of a

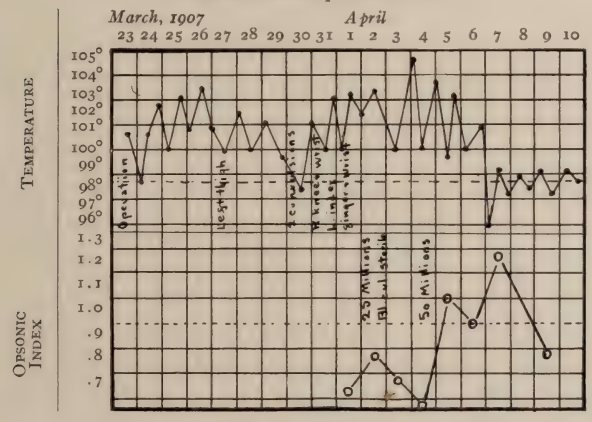
vaccine, this patient would have been inoculated and his subsequent recovery would doubtless have been attributed to its use.

Case 4.—A. H. Female. 58. Dr. A. J. Bristow and Dr. W. H. Pratt, Brooklyn. February 25, rigors, fever, general muscular pains, intense headache. March 1, well. March 4, repetition of previous attack with more severe headache. March 10, well. March 11, tenderness over left side of neck. Fever, temperature 100° to 103°. March 17: moderately circumscribed swelling of left submaxillary triangle. No fluctuation. Leucocytes 15,000, polynuclears 78 per cent. March 18, swelling less painful, less tender. Leucocytes 12,000, polynuclears 78 per cent. March 20, swelling more diffuse, more painful. March 21, leucocytes 10,000, polynuclears 75 per cent. March 23, swelling had extended to clavicles, more tender. Leucocytes 11,000, polynuclears 83 per cent. A long incision parallel with anterior border of sternocleidomastoid, tissues much thickened and infiltrated. Deep in submaxillary fossa small abscess cavity opened, pus evacuated, drainage. Culture: *Strept. longus*

CHART 3.

MRS. H. STREPTOCOCCUS ABSCESS IN NECK WITH MULTIPLE ARTHRITIS.

Inoculated with streptococcus from A. R.



and a bacillus. March 24, edge of wounds glazed. Very little discharge. Urine: normal. March 26, intermittent pulse. March 27, severe pain in left leg. Some tenderness over internal condyle. March 28, infiltration of neck slowly disappearing. March 30, two slight convulsions limited to face and upper extremities, eyes to right. Face livid. Blowing murmur over mitral. March 31, pain in right knee and left forefinger and wrist. April 1, seen by Dr. Potter. Opsonic index to streptococcus A. R. (Dr. Krumwiede), 0.74. April 2, blood culture (Dr. N. E. Ditman) sterile. Inoculated with 25 millions streptococci A. R. (Vaccine prepared by Dr. Krumwiede.) April 4, wound diminished in size one-half. Joints have improved. Inoculated with 50 millions. April 11, wound practically healed. Convalescence uninterrupted. The striking features of this case are the improvement of the wound and joint symptoms within 48 hours after the first dose, and the sudden drop in the temperature and rapid improvement following within 48 hours after the sec-

ond inoculation and closely corresponding to the rise in the opsonic index, as plainly shown in the chart.

The question whether two sets of leucocytes give indices which are nearly alike (other conditions being the same) was investigated. The possibility of an altered power of phagocytosis in an infected person's leucocytes, was considered. In the later experiments the number of polynuclear neutrophiles (phagocytes) in the cream were counted as it was thought that the variations in the phagocytic average might coincide with the variations in the number of leucocytes. With fewer cells, other conditions being the same, one would expect more cocci to each cell. In other words, the phagocytic average would vary inversely as the number of leucocytes.

The following table shows our results and requires no explanation. Although no conclusions can be drawn from so few observations, especially when the results depend upon so inaccurate a method of technique, yet they seem to suggest, just as do similar observations upon the staphylococcus and the tubercle bacillus infections that the leucocytes of patients with pneumonia during the height of the disease are probably less active in phagocytic power than normal leucocytes. During and just after the crisis or lysis their phagocytic power increases rapidly, surpasses, and then later equals or becomes less than that of normal leucocytes.

MacDonald[1] in London examined 25 cases of acute croupous pneumonia and 17 cases of broncho-pneumonia. Although his figures are derived from many more experiments and show more striking variations, his results in the study of the opsonic index in pneumonia are practically the same as ours.

Wolf[2] in Chicago has also arrived at similar conclusions from the study of 11 pneumococcal infections, five of which were cases of typical lobar pneumonia terminating in crisis, two were fatal cases of pneumonia, two were migratory pneumonia and two were pneumonic empyema. He suggested the estimation of an antipneumococcal index by comparing the leucocytic index and the opsonic index and found this to be increased early in pneumonia, and to remain high until crisis was completed in cases with favorable terminations. Neither

[1] *Aberdeen University Studies*, No. 21, 1906.

[2] *Jour. Infect. Dis.*, 1906, 3, p. 731.

TABLE 1.

Condition of Patient Whose Leucocytes Were Used	Date	Phagocytic Average		Ratio in Per Cent	Opsonic Index		Serum	No. of Polynuclears in 1 c. mm. of Leucocytic Cream		Ratio (Inverse) Per Cent	Organism
		Normal Leucocytes	Patient's Leucocytes		Normal Leucocytes	Patient's Leucocytes		Normal	Patients		
Lobar pneumonia 10th day terminating empyema	Feb. 5	2.26	1.17	0.52	0.94	1.20	F. R. Pneum.	.....	.....	....	Pneum.
		1.96	1.05	0.54	....	....	F. R. Pneum.				"
		2.37	0.81	0.34	....	....	Normal				"
		2.11	0.94	0.45	....	....	"				"
Broncho-pneum. Convalescent	Feb. 19	3.57	2.31	0.66	1.25	1.20	J. B. Pneum.	.....	.....	....	Pneum.
		2.94	2.03	0.69	1.03	1.03	M. L. R. Pneum.				"
		2.84	1.96	0.70	....	....	Normal				"
Streptococcus Septicopyemia	Feb. 23	1.69	1.13	0.67	1.02	0.91	A. R. Sept. Pyem.	16,107	18,088	0.89	Pneum.
		1.49	0.92	0.61	0.90	0.74	F. R. Pneum.				"
		1.80	1.19	0.66	1.09	0.98	M. R. L. Pneum.				"
		1.65	1.24	0.75	....	....	Normal				"
Same, extremely marked improvement, temp. falling	Mar. 5	4.61	5.97	1.30	1.24	1.29	M. R. Pneum.	13,724	12,853	1.06	Pneum.
		4.92	7.90	1.61	1.33	1.69	M. L. R. Pneum.				"
		3.70	4.64	1.25	....	....	Normal				"
Same. Convalescent	Mar. 25	1.43	1.85	1.26	1.07	1.02	A. R. Sept. Pyem.	11,850	11,440	1.03	Pneum.
		1.33	1.80	1.35	....	....	Normal				"
		5.31	4.68	0.88	1.01	1.02	A. R. Sept. Pyem.				Strep. A. R.
		5.24	4.58	0.87	....	....	Normal				" "
Lobar pneum. resolution, beginning strep. pleurisy	Feb. 28	5.33	7.95	1.49	1.50	1.33	M. L. R. Pneum.	19,712	24,825	0.79	Pneum.
		5.42	7.33	1.33	1.51	1.22	Q. R. Sept. Pyem.				"
		3.37	4.95	1.46	0.94	0.82	M. R. Pneum.				"
		3.57	5.98	1.67	....	....	Normal				"
					1.51	....	M. L. R. Pneum.				Strep. M. L. R
Same. Cured	Mar. 29	3.01	3.06	1.01	0.93	0.91	M. L. R. Pn+Pleur.	9,940	9,652	1.03	Pneum.
		3.22	3.34	1.03	....	....	Normal				"

of these two observers has noted the variability in the phagocytic power of leucocytes from patients with pneumococcal infections as compared with leucocytes from normal individuals.

NOTE.—Shortly before this article was put into type, my attention was called to Rosenow's studies upon the phagocytic power of pneumonic as compared with normal leucocytes (*Jour. Inject. Dis.*, 1906, 3, p. 683). These studies had escaped my notice. His observations were much more carefully conducted than ours, in that the leucocytes from each source were very thoroughly washed in normal saline and their numbers equalized in each cream. He does not state whether, as we did, he took into account the proportions of polymorphonuclears or not. His conclusions were that (1) pneumonic leucocytes are more resistant to heat than normal leucocytes; (2) when heated just sufficiently to lose their phagocytic power they are more positively chemiotactic; (3) they not only take up pneumococci which are freely susceptible to phagocytosis in large numbers, but take up strains that resist phagocytosis by normal leucocytes; (4) they are more actively phagocytic for certain rare strains of pneumococci in the absence of serum.

Our observations suggest a decreased phagocytic power before, and an increased during and after, the crisis. Rosenow, on the contrary, found an increased phagocytic power of all pneumonic leucocytes without reference to the stage of the disease or convalescence.

OBSERVATIONS UPON CERTAIN PROPERTIES ACQUIRED BY THE PNEUMOCOCCUS IN THE HUMAN BODY.*

LEO BUERGER AND CHARLES RYTTENBERG.

(From the Pathological Laboratory, Mt. Sinai Hospital, New York.)

THE study of a large number of strains of pneumococci in the course of the last year's[1] routine bacteriological work led us to make certain observations upon the acquisition of new cultural properties by these organisms in the human body. In previous publications[2] one of us pointed out the variations that the pneumococcus may present in its morphology and in its cultural characteristics. The value of morphology in diagnosis was discussed as well as the value of the appearance of the colonies (ring colonies), the inulin and the glucose-serum media. Our experience led us to attach considerable importance to the typical morphology to which we applied the term "pneumococcus type." As regards the cultural properties it was found that although most pneumococci fermented inulin, certain streptococci also possessed this power, and that some pneumococci failed to break up this carbohydrate in every generation; further, that whereas streptococci caused precipitation in the glucose-serum-agar, only a very few pneumococci show this phenomenon; and finally, that streptococci usually produced hemolysis in blood media, but pneumococci either did not or only caused it in the blood in which they were found.[3] Thus, although not a single cultural property is differential between the streptococcus and pneumococcus, we may speak of a streptococcus and a pneumococcus cultural type, in order to facilitate future discussions. The streptococcus cultural type would include the following: non-fermentation of inulin, precipitation of glucose-serum-agar, and hemolysis in blood media; the pneumococcus cultural type, fermentation of inulin, non-precipitation, and failure to produce hemolysis.

* Received for publication October 10, 1907.

[1] 1906–1907.

[2] *Centralbl. f. Bakt.*, 1905, Orig. 39, pp. 216, 335, and *Jour. Exp. Med.*, 1905, 7, p. 545.

[3] LIBMAN, *Johns Hopkins Hospital Bulletin*, 1906, 17, p. 215.

While studying the blood of a case of puerperal pneumococcemia we isolated pneumococci differing from the ordinary type in that they failed to ferment inulin and caused precipitation of glucose-serum-agar (streptococcus cultural type). Upon further investigation we found that it was possible to convert this organism into the ordinary variety by animal inoculation. This led us to study carefully other pneumococci recovered from human exudates and blood, to investigate particularly those strains which varied from the type, and to attempt to effect a reversion to the normal by inoculation into susceptible animals. It is our purpose to record briefly a number of our observations.

OBSERVATIONS UPON CASE I.

Puerperal pneumococcemia; streptococcus cultural type in three successive blood cultures and in a metastatic abscess; conversion into pneumococcus cultural type by passage through mice; failure to change properties of strain isolated from metastatic abscess.

1. Blood culture 863: March 18, 1907. Colonies on blood plates surrounded by zone of hemolysis. Morphology: chains with "streptococcus type" capsule. Inulin, negative;[1] precipitation,[2] positive.

2. Blood culture 870: March 21, 1907; same as above.

3. Blood culture 882: March 29, 1907; taken two days ante mortem. Colonies: some ring forms; hemolysis distinct. Morphology: like degenerate pneumococci; streptococcus type capsule. Inulin negative; precipitation positive.

Culture 9,729, from metastatic abscess in anterior and posterior chambers of eye. Morphology: typical pneumococci. Colonies: ring form. Inulin negative; precipitation positive.

Animal Experiments, April, 1907.

Culture 882*a:* in mouse 22, April 7; died in three days. Typical pneumococci in blood. 882*b* isolated.

Culture 882*b:* typical pneumococcus; inulin positive; precipitation negative.

Culture 882*a*, in mice 24 and 25; both died after 24 hours. 882*c* and 882*d* isolated.

Cultures 882*c* and 882*d:* typical pneumococcus morphology and cultural type.

Culture 9,729: in mice 23 and 28; mouse 23 killed in 5 days, negative; mouse 28 died in 2 days. 9,729*a* isolated; inulin negative; precipitation positive. Successive inoculations into mice; same result.

Animal Experiments, May, 1907.

Culture 882*a:* in mouse 48, May 7, died in 3 days; pneumococcus of typical morphology in blood. Streptococcus cultural type. Several other mice inoculated with same result. Hence the organism cannot now be converted to pneumococcus cultural type.

Culture 9,729 in a number of mice; remains streptococcus cultural type.

[1] Negative means not fermented.

[2] Precipitation refers to whitening or precipitation in glucose-serum-agar.

Animal Experiments, June, 1907.

Cultures 882*a* and 9,729 into several mice on June 6 and June 24. The organisms remained of the streptococcus cultural type.

Here we have a significant instance of the labile nature of the pneumococcus and of the mutability of its fermentative properties. The organisms from the blood possessed the cultural features and even the morphology of a streptococcus. A metastatic focus contained organisms of the typical pneumococcus morphology but otherwise resembling streptococci. It is interesting to note that three of the strains reverted to the normal type after passage through white mice, but that one organism from the eye focus retained its peculiar properties. After the lapse of one month it was found impossible to bring about a change to the normal type in any of the cultures and even those that had been converted assumed their original features. After three months the streptococcus cultural type seems to have become fixed, not only in the original strains but also in those that had for a time taken on the usual cultural features of pneumococci.

OBSERVATIONS UPON CASE 2.

Chronic endocarditis, lobar pneumonia and pneumococcemia. Streptococcus cultural type; conversion into pneumococcus cultural type.

Organisms studied: (*a*) and (*b*) blood cultures; (*c*) postmortem, lung culture; (*d*) postmortem, spleen culture.

Culture (*a*) morphology typical pneumococcus; colonies large, white on plates, surrounded at first (24 hours) by green area, later by zone of hemolysis. Inulin negative; precipitation positive.

Cultures (*b*), (*c*), and (*d*), same as (*a*).

Culture (*b*) inoculated subcutaneously into two white mice; death in 24 and 48 hours respectively. Two strains isolated b_1 and b_2.

Strain b_1, pneumococcus cultural type.

Strain b_2, streptococcus cultural type.

In this instance we were dealing with pneumococci that had acquired the cultural features of streptococci in the human body. Passage through a white mouse effected a complete change as regards inulin fermentation and precipitation of glucose-serum-agar.

OBSERVATIONS ON CASE 3.

Acute osteomyelitis; pneumococcus with typical capsules and streptococcus cultural properties; it remained unchanged after repeated animal inoculation; a later culture obtained at a second operation showed typical pneumococci both as to morphology and cultural characteristics.

Culture (*a*) from subperiosteal exudate. Culture (*b*) from marrow cavity. Cultures identical. Morphology: a few diplococci and many short chains; they are of the pneumococcus type, but somewhat mucoid, inulin negative; precipitation positive.

Animal experiments.

Mouse 33, April 15, 1907; died in 48 hours. Cultural properties unchanged, still of streptococcus type. This strain was inoculated into mouse 35 and the organism isolated was again passed through mouse 37. No change in properties was thus effected. On June 6 and 24, all the strains were again tested and still found to possess the streptococcus cultural type.

Culture (*c*) isolated *two and one-half weeks later* at a second operation. *Typical pneumococcus*—both in morphology and culture.

Another variation different from the preceding presented itself to us in this case. The difference in the cultures obtained at the first and second operations are interesting. The first organisms isolated retained their streptococcus cultural properties despite successive animal inoculations whereas the second strain derived from the same source two and one-half weeks later was a typical pneumococcus.

OBSERVATIONS ON CASE 4.

Retropharyngeal abscess; pneumococcus of the mucoid type[1] *with typical morphology, but possessed of the streptococcus cultural type; failure to convert it into the pneumococcus cultural type by repeated animal inoculations.*

Culture 9,838: typical pneumococcus morphology; inulin negative; precipitation, positive.

Animal Experiments.

Mouse 31, inoculated April 11, 1907; remains of streptococcus cultural type. Mouse 36, April 23, ditto. Otherwise gave same result. June 6 and 24—animal inoculations; streptococcus cultural type persists.

This organism presents still another variety, namely a mucoid pneumococcus that rapidly lost its luxuriant growth, and that failed under any circumstances to ferment inulin. Such pneumococci are the most difficult to classify, for it is only by their morphology that they can be recognized. Possibly some bacteriologists would regard them as atypical streptococci rather than pneumococci. We are inclined to look upon this strain as one that had acquired new cultural features in the human body, and had retained its morphological characteristics.

[1] BUERGER, *Jour. Exp. Med.*, 1905, 7, p. 497.

In view of the fact that it was found possible to convert certain strains from the atypical into the typical variety by means of animal inoculation, it seemed of practical as well as of theoretical interest to determine whether the same result could be obtained by the use of favorable media; and, vice versa, to inquire into the action of human blood on the typical organisms with a view to seeing whether it would cause the same changes in the cultural features that were found to take place in the human body.

Pure sterile ascitic fluid exerted a marked effect upon certain strains; indeed, a 24-hour growth in this medium often produced large encapsulated forms which grew luxuriantly for a number of generations. However, the power to ferment inulin could not be restored by its use, and the influence of this medium was confined to a temporary enhancement of growth and a change in morphology.

A number of typical pneumococci were grown in sterile human blood in which coagulation had been prevented by an admixture of sterile ammonium oxalate solution 0.2 per cent. The blood was obtained by aspiration from one of the veins of the forearm, and about 3 to 5 c.c., mixed with an equal amount of ammonium oxalate, were used for each test. It had been previously found by Bernstein and Epstein[1] that the ammonium oxalate does not interfere with the growth of most of the pathogenic organisms. Subcultures were made after intervals of 3, 4, 5, and 7 days and the organisms were tested on inulin and glucose-serum-agar. It was not found feasible to convert a pneumococcus cultural type into the streptococcus variety by this means.

Another point which seemed worthy of investigation was the possibility of effecting a change in the cultural properties of atypical organisms after the lapse of several weeks or months. With this end in view the strains of the streptococcus cultural type were again passed through white mice after periods of about two and three months. Two months after isolation all the atypical organisms had apparently acquired fixed streptococcus cultural characteristics and repeated animal inoculation failed to bring about a change. The same negative result was obtained when the experiments were repeated one month later.

[1] *Jour. Infect. Dis.*, 1906, 3, p. 772.

GENERAL SUMMARY.

Observations on the morphology and cultural features of pneumococci in human exudates and human blood lead us to the conclusion that wide variations from the type may occur. They tend to point rather significantly to the influence that the human body may exert upon the fermentative properties of these organisms. From a consideration of the variations from the type and from the results of animal experiments we must needs become impressed with the mutability of the fermentative properties of the pneumococcus and become even more careful in attaching importance to fermentation tests in diagnosis.

In summing up the results of our studies we wish to call attention to the following points:

Pneumococci may acquire certain unusual cultural properties in human blood or in human exudates. The power to ferment inulin may become lost, and the ability to cause precipitation in glucose-serum-agar may be gained. In other words a substitution of the common cultural features of pyogenic streptococci may take place; the organisms then resemble the "streptococcus cultural type."

Although many strains fail to cause hemolysis in blood agar, the organisms isolated from the circulating blood frequently cause hemolysis when grown in the blood of the host. .

Pneumococci in the blood of a patient suffering from a general infection with this organism may differ in cultural characteristics from those obtained from the metastatic foci.

The cultural properties of pneumococci derived from the same source and isolated at different periods in the course of the patient's disease may vary, for at one time we may see the typical pneumococcus morphology associated with the streptococcus cultural type, at another, we may note typical pneumococcus morphology and pneumococcus cultural features.

Pneumococci of the streptococcus cultural type may be possessed either of the morphology of typical pneumococci or of ordinary streptococci.

It is possible to convert some but not all strains possessing the streptococcus cultural type into typical pneumococci by inoculation

into white mice. All of the pneumococci isolated from one case may not permit of this change.

The morphology of pneumococci as it appears with the capsule stain proposed by one of us[1] may alone give a clew in the establishment of a diagnosis. By means of the capsule stain we were sometimes able to make a diagnosis several weeks before other substantiating evidence could be obtained. Thus one strain (from a metastatic focus in a case of pneumococcemia), which was diagnosticated as pneumococcus because of its typical morphology (Observation 1) was found to be possessed of streptococcus cultural features; and failed to permit of conversion to the normal type by animal inoculation. In still another instance (Observation 3) where we were dealing with an organism from a case of acute osteomyelitis, the morphology was that of a typical pneumococcus. All the other features, however, were of the streptococcus type and animal inoculations proved useless. Here later cultures proved conclusively that the organism in question was pneumococcus.

The tendency of pneumococci of the streptococcus cultural type as well as those which have been converted to the normal variety, seems to be toward a gradual degeneration which manifests itself in the assumption of *permanent* streptococcic features. Such organisms can then no longer be differentiated from streptococci.

For purposes of diagnosis we deem the following conclusions worthy of consideration:

1. The fermentation of inulin is of limited value in the differential diagnosis between pneumococci and streptococci. Pneumococci may lose the power to produce acid in this carbohydrate either temporarily or permanently.

2. The precipitation of glucose-serum-agar although more frequently a property of streptococci, may also be produced by the action of pneumococci.

3. Hemolysis in blood agar may be caused by both streptococci and pneumococci.

4. The morphology of pneumococci as it appears with the capsule stain employed by us frequently allows a diagnosis to be made when

[1] *Centralbl. f. Bakt.*, 1905, 39, pp. 216.

all other means fail. Whenever an organism presents the typical pneumococcus capsule together with the cultural features of a streptococcus, we must have recourse to animal inoculation with a view to converting the organisms into the normal variety. When this fails, the diagnosis, although not absolutely certain, may, according to our experience, be safely made in favor of the pneumococcus.

A CASE OF VEGETATIVE ENDOCARDITIS, CAUSED BY AN UNIDENTIFIED PLEOMORPHIC BACTERIUM.*

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The case here described is of interest in that the inciting micro-organism, which was present in the blood during life, presents rather remarkable characteristics, and differs, we believe, from any of the bacteria hitherto found in association with endocarditic lesions.[1]

Clinical Record.

The patient, aged 44 years, a janitor, was admitted to the hospital July 28, 1906, and was under observation until his death in the hospital November 28, 1906.

Previous history.—Has lived in New York City for 41 years and has worked as a janitor for 1½ years; clerk for 10 years before this. Heavy drinker of beer and whiskey, with frequent excesses. Chews excessively, but smokes only occasionally. One cup of coffee daily. Eats irregularly. Sleeps poorly. Bowels move once a day. Usual weight is 135 lbs. Scarlet fever when a child; good recovery. German measles at 20 years. Fifteen years ago was in a hospital for 11 days, suffering from nervousness, insomnia, and vomiting (acute alcoholism?). Twenty-four years ago had a urethral discharge and pain on urination, lasting three months. At this time he had four sores on the penis which discharged yellow material. Under treatment these healed within a week. During the ensuing year, he had two similar attacks, with multiple sores on his penis. No history of secondary symptoms.

Present illness.—Dates back two weeks. Patient awakened one morning with dull pain in both hips; when he started to walk he also felt pain on the inner side of his left thigh. This has continued, and he has also felt feverish. No other pain. Appetite is good, bowels regular. There is no dyspnoea. For past year and a half he has had a cough at times, never accompanied by much sputum. He has had about a half-dozen night sweats. He believes he has lost some weight of late. Urination normal. No recent urethral discharges. Pain in thighs and fever are chief complaints. Upon admission: T. 103°; P. 126; Resp. 22.

Physical examination.—Patient is of medium frame, fairly well nourished and developed. Skin is pale, warm, and moist. Mucous membranes somewhat pale. No cutaneous eruptions. Lymph-nodes palpable, not enlarged. Pupils equally contracted and react normally. Tongue is narrow, pointed, moist, clean, slightly tremulous. Pulse small, slow, of good tension, regular; radial artery somewhat thickened. Apex beat not visible or palpable. Heart sounds heard with maximum intensity in 5th space, 3½ inches to left of median line. First sound at apex accompanied by a blowing murmur, transmitted into axilla. Basic sounds indistinct; no murmurs

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[1] We are very greatly indebted to Drs. A. A. Smith, E. LeFevre, and R. J. Carlisle, visiting physicians to the Third Medical Division of Bellevue Hospital, for permission to transcribe the clinical records here presented.

heard. Chest well developed, expansion good. Lungs within range of normal. Liver, dullness at 4th space, flatness at 6th, extending a finger's breadth below costal margin. Edge of liver not palpable. Spleen not palpable. Abdomen not distended. No fluid, tumors, or tenderness. Tenderness on deep pressure behind trochanters; also along the course of the sciatic nerve. Patellar reflexes normal.

August 2. Since admission, temperature has been remittent between 99° and 102.+°. Pulse 80–90. No pain in back. General condition fair. Slight cough with purulent sputum. No tubercle bacilli in sputum. Yesterday and today, a few fine crackling râles heard on inspiration over lower part of left chest in anterior axillary line (5th space). Liver palpable a finger's breadth below costal margin—slightly tender.

August 6. Since last note, temperature has been irregular, ranging between 100° and 102°. Lungs negative. Systolic murmur heard all over precordial region. Maximum intensity in 3d left interspace near sternum. No pain or limitation of motion in hips. General condition fair. Still has a slight cough with scanty mucoid expectoration.

August 8. First blood culture taken: sterile.

August 14. Today, temperature dropped from 102.6° to 99°. Complains of severe pain and tenderness in calves of legs.

August 15. Temperature rose to 102.6°. Lungs negative. Pains in legs persist.

August 16. Today, patient had a distinct chill lasting 10 minutes, after which temperature rose to 102.2°. Lungs normal. Some tenderness in umbilical region. Examination of blood for malarial plasmodia negative.

August 21. Since last note, temperature has ranged between 99° and 101°. Pulse 78–96. General condition fair. Complains of some tenderness in both calves. No edema. Murmurs over precordium have changed. Rough systolic murmur is heard at 2d right interspace, not transmitted. A similar murmur at apex, not transmitted. Lungs negative. Had a slight chill last night.

August 24. Since August 21, temperature has been somewhat higher; shows daily remission between 100° and 102°. Very cyanotic today. Profuse sweating. No pain in legs. Bowels constipated. Cardiac condition unchanged.

August 28. Temperature still irregular, ranging between 99° and 102°. Cardiac condition unchanged. Lungs negative. Spleen barely palpable. Abdomen not distended, not tender. No tenderness in legs. No chill. No eruption. Slight cyanosis of fingers and lips in mornings.

September 11. Since last note, temperature has ranged between 98° and 101°. Today patient has had chilly sensations, but no distinct chill. Very cyanotic. Complained of aching pains in shoulders. Cardiac condition unchanged. Lungs negative. Spleen palpable.

September 17. Second blood culture taken: sterile.[1]

September 24. Temperature, pulse, and respiration continue about the same. Cardiac signs unchanged, save that murmurs are more intense. Systolic murmur present over apex has been louder during past few days than on previous note. Systolic murmur over 2d right interspace persists. No pericardial murmurs heard. Lungs: a few scattered moist râles over both bases. No change in voice sounds.

[1] A Gram decolorizing bacillus was obtained from one of the flasks, but subcultures failed to grow. Organism was considered at the time as being probably a contaminating growth, and the culture reported as sterile.

Appetite fair. Patient says he feels weak. No skin eruption has been noted at any time. Patient complains of some stiffness in left wrist upon awaking in the morning. No pain or tenderness in joints. Has some pain in the abdomen, which is dull in character. There is moderate distension. Spleen is still palpable and slightly tender. Stools formed, very dark. No ova present.

September 28. Since last note, T. P. R. has ranged between the limits stated on previous notes. T. = 98°–100.6°; P. = 72–90; R. = 18–24. Patient cyanotic in the morning. Has indefinite pains in arms and legs. Left wrist slightly swollen and tender. Motion limited. No change in cardiac condition. Lungs normal. Spleen still palpable—not tender. Liver not palpable. No skin eruption. No chills, chilly sensations, or night sweats. Appetite fair. Feels very weak, and has lost flesh.

October 4. For the past four days, range of temperature has been higher, reaching 101.2° and dropping to normal. No chills, chilly sensations, or sweats. Patient is still cyanotic in the mornings.

Systolic murmurs over apex and base still present. Apical murmur is intense, transmitted into left axilla and heard behind between scapulae. Lungs negative. On October 2, patient complained of dull pains in both shoulders and arms—had no tenderness or swelling. Appetite fair, sleeps fairly well.

October 10. Temperature intermittent: 98°–100.8°. Pulse 80–84; regular. Respiration 20. Patient feels comfortable. Cyanosis, and stiffness in left wrist continue. Heart unchanged. Lungs: breath and voice sound distant over both bases. No râles heard. No tenderness along course of nerves in extremities. No edema about ankles. No eruption. Weight not taken, as patient has been continuously in bed since admission. Medication: salicylates, oil of wintergreen, tonics.

October 16. Pain in left wrist not so severe as before. T. P. R. same as on previous notes. Heart murmurs unchanged. No petechiae. No edema of ankles. Bowels constipated.

November 8. Temperature is still remittent in character, ranging between 99–103°. Complained of feeling chilly yesterday; no distinct chill. Physical condition unchanged.

November 10. Indistinct pains in left wrist. No limitation of motion or tenderness.

November 15. Complains of aching pains with some tenderness of left wrist. Cardiac action regular. Systolic murmur at apex is louder. Rumbling diastolic murmur is heard at apex. No thrill felt. At base over aortic area, systolic still present. Faint diastolic murmur heard here and over 3d left space near sternum.

Lungs show scattered moist râles. Spleen still palpable. Fourth blood culture taken: Positive. (See bacteriological report.)

November 18. Patient complains of numbness in whole of left lower extremity. T. is 102.4°. P. 108. R. 20. No edema; no ecchymosis; no eruption.

November 19. Still complains of numbness of whole left lower extremity with pain about left hip. Examination shows no edema; no pulse is obtained over dorsalis pedis artery. Extensive ecchymosis from middle of left thigh to middle of leg. Considerable tenderness below inner half of left Poupart's ligament. Some induration made out. Cardiac action regular. Pulse 96–100. Double murmurs at base and apex still present, but not quite so distinct as on November 17 and 18. No thrill made out at apex. Lungs show few scattered moist râles. Fifth blood culture taken: positive. (See bacteriological report.)

November 20. T. 102°; P. 102; R. 22. Ecchymosis of left leg more marked sharply limited at middle of thigh. Marked change in surface temperature, i. e., from warm to cold, at line of demarcation. Sensation is entirely lost below this level. No edema. Almost complete paralysis of left lower extremity. Tenderness and induration extend along the course of the femoral vessels from Poupart's ligament to line of ecchymosis. No redness along this area. Some tenderness behind left greater trochanter. Lung signs unchanged. Heart regular. Blowing diastolic murmur at 3d left space and at apex is much louder. Reduplication of 2d sound at apex is present at times.

November 21. Patient delirious for the first time. Marked general cyanosis. Condition of left lower extremity unchanged. Cardiac murmurs unchanged; action regular. Lungs normal, except for scattered râles.

November 22. Temperature fell from 102.8° at 8 P. M. on November 21 to 97.2° at 8 P. M. on November 22. Pulse 98–112; regular. Still very delirious, general cyanosis. Ecchymosis has extended along inner side of left thigh from its middle to Poupart's ligament. Tenderness in thigh is still marked. Some tenderness and a muscular rigidity is noted in left iliac region of abdomen. Along the lowest inch of the course of the left radial artery, there is induration with apparently slight tenderness. No pulse is felt. Few small petechiae have appeared over left elbow.

November 23. T. P. R., 99°—96—24. Still delirious. Cyanosis is moderate. Induration along left radial artery unchanged. Condition of left lower extremity unchanged. Apparently has severe pains in left leg. All the toes of right foot are cold and very cyanotic. No pulse wave felt over right dorsalis pedis and posterior tibial arteries.

Cardiac action regular. Murmurs unchanged. Lungs negative, except for scattered râles.

Abdomen slightly distended. Spleen palpable. Tenderness and rigidity in left iliac region still present. No new petechial spots. Cyanosis of right foot, sharply limited above webs of toes on dorsal and plantar surfaces.

Vomited coffee-ground material yesterday. Slight cough; no expectoration.

November 24. Moderate general cyanosis. Delirium persists. Cardiac signs unchanged. Lungs scattered moist râles. Abdomen slightly distended. Tenderness in lower left quadrant.

Condition of right foot and left lower extremity unchanged. Left radial pulse not made out. Induration along the lowest inch of its course in forearm still present. Skin over this area slightly reddened.

November 25. Patient died this A. M.

Urinalyses.—Examinations of the urine were made at intervals of a few days throughout his stay in the hospital. The total daily amount ranged between 30 and 56 ounces. A faint trace of albumin and a few hyaline and granular casts were occasionally noted. The specific gravity averaged low (1.013), and the urea excretion was uniformly diminished.

The sputum was examined on nine different occasions. No tubercle bacilli were found.

Examination of the feces on several occasions showed nothing of interest.

POST-MORTEM RECORD.

Necropsy performed by Dr. Norris, 9½ hours post-mortem.

General appearance.—Body light, weight about 90 lbs., height 5 ft., 6 in., ema-

TABLE 1.

Blood Examinations.

Date	Erythrocytes	Hemoglobin	Leucocytes	Polynuclears	Lymphocytes		Eosinophiles	Transitionals and Large Mononuclears	Remarks
					Large	Small			
July 29	.....	....	17,200	73	5	14	1	7	No plasmodia
" 28–31	.....	....	.....	..	...	....	...	...	" "
Aug. 1	.....	....	24,000	80	5	2·5	0	2·5	.....
" 4	.....		17,300						No plasmodia
" 7	.....		18,400	72	6	13	2	7	
" 16	.....		16,600	85	7	4	0	4	No plasmodia
" 24	.....	....	20,000	..	...	....	...	...	
" 29	4,600,000	60%	14,400	77	5·5	7	1·5	8	Basoph. 1%
Sept. 8	.....	....	24,600	..	...	....	...	...	.....
" 18	.....	....	21,000						
" 25	.....	....	14,000						
Oct. 1	.....	....	18,000						
" 4	4,750,000	65%	.....	..	...	....	...	...	
" 10	.....	....	15,000	..	...	....	...	...	
" 20	.....	....	12,000						
" 30	.....	....	15,000	80	4	12	...	4	
Nov. 15	.....	....	15,000	80	6	13	0	1	
" 19	.....	....	12,000						
" 20			13,200	81	3	13	0	3	
" 24	.....	....	26,600	82	5	4	0	9	

ciated, slight general icterus. The toes of the right foot, except the big toe, show bluish discoloration, and the epidermis has peeled. The toes of the left foot (2d, 3d, and 4th) are slightly reddish in color; otherwise normal. Above the left knee for a distance of nine inches, there is an extensive bluish ecchymosis. No edema of the legs. Left thigh slightly larger than the right. Slight P. M. suggillation of back and shoulders. Rigor mortis marked. On the left elbow, externally, a few petechial spots, skin over which is desiccated. Muscles are yellowish red in color, and appear somewhat friable.

Diaphragm 5th left space, 5th right rib.

Liver. Right lobe at the free margin of ribs. Left lobe extends about 4 in. beneath the ensiform cartilage.

Peritoneal cavity contains a few drachms of bile-stained fluid in the recto-vesical fossa. Ribs are not ossified.

Lungs.—No adhesions or fluid in the left pleural cavity. Both lungs emphysematous. Left lower lobe somewhat firm posteriorly. In the upper part of the lower lobe, a small reddish infarct is present. On section, the lower lobe, is slightly granular, and in places there are small areas of lobular pneumonia.

Bronchi are slightly hyperemic, congested, and contain yellowish, frothy fluid in small amount. Bronchial lymph nodes are anthracotic, without evidence of tuberculosis.

There are about 750 c.c. of turbid bile-stained fluid in the right pleural cavity.

There is a considerable amount of ochre-colored fibrin between the fissures of the lobes of the right lung, which are more or less adherent. Lower lobe feels solid. On section it has a mottled appearance, granular on section and dry, and in places contains a few small yellowish foci. At the base, there is a reddish area, grumous on section, projecting from the surface (infarct). The lower half of the lobe posteriorly, is in a condition of red hepatization, section being slightly grumous. The rest of the lung is normal.

Heart.—Pericardium contains about an ounce of extremely turbid yellowish fluid. The pericardium itself is in places slightly injected, and there are a few yellowish, small fibrin floccules. Right auricle, especially the ear, is intensely injected and thickened. Right heart is somewhat distended with dark reddish blood clots. The pulmonary artery and valves are normal. Tricuspid ring admits three fingers and

FIG. 1.—Heart, reduced one-third, showing aortic vegetations.

is normal. In the right auricle, there is a softened puriform thrombus, which is firmly adherent to the auricular wall. The central portion of the clot shows whitish softening. Mitral orifice admits two fingers, the valve is slightly thickened at the edges, but is otherwise normal. Left ventricle of normal thickness. Right ventricle slightly stiff, but scarcely hypertrophied. Muscle is red and normal in appearance. Left auricle is normal. Its endocardium, and the endocardium of the left ventricle. is slightly opaque. The three aortic cusps are markedly thickened and adherent. On

section, they are in places almost 1 cm. in thickness and are composed of a firm, dry, slightly yellowish material, in several places perhaps chalky. The left posterior cusp, at its base, shows a small tear or ulceration. The right posterior is the seat of a firm mass of tissue, and in the sinus above this cusp there is a reddish clot, which is adherent to the firmer tissue, resembling that found on the cusps. The sinus shows an aneurysmal dilatation or pouch, which admits a small marble, say 25 mm. in diameter. The aorta itself is perfectly normal. The heart as a whole is somewhat enlarged.

In the left common iliac, about 2 cm. below the bifucration, there is a firm, adherent reddish thrombus, which completely occludes the vessel, and extends beyond its bifurcation. The external and internal iliac arteries contain a soft, reddish thrombus, which is partially adherent.

Spleen.—Adherent to the diaphragm. Weight 205 gms. Spleen is soft and contains an infarct, triangular on section, measuring 15×20 mm. There is a deep scar in the upper pole of the organ.

Pancreas.—About normal in size, and firm.

Suprarenals.—Normal.

Kidneys.—Moderate amount of perirenal fat. Left kidney weighs 105 gms. Small and firm. Cortex thin, markings indistinct. Capsule adherent, leaving a slightly granular surface. There are a number of old scars, which extend deeply through the cortex. Kidney is extremely pale.

Weight of right kidney, 150 gms. In the upper pole, there is a mass of firm tissue, which presents a deep ochre-yellow color, about 8 mm. in length, and extending from the cortex well into the pyramid. There are likewise a number of smaller yellowish areas adjacent to it, the rest of the kidney being similar to the left. There are a few small cysts.

Ureters and urinary bladder normal.

Prostate and testicles and epididymes normal in size and to the touch, but were not examined.

Liver.—On pressure, no bile exudes from the papilla. Weight 1,380 gms. Surface is slightly granular, yellowish, and cuts somewhat gritty. On section color intensely yellowish and lobulations indistinct. Gall bladder contains sticky, tarry bile.

Esophagus is normal. One of the perigastric lymph nodes is hyperemic.

Stomach.—Contains an excessive amount of mucus, otherwise normal. Abdominal and thoracic aorta normal.

Intestines.—The duodenum and the upper part of the jejunum are normal. For a distance of approximately eight feet, small intestine is intensely congested. The mucous membrane is thick, and is covered with a whitish, often distinctly yellowish exudate, and in places it has the appearance of being infiltrated with pus. No superficial erosions.

The intestinal canal at this point contains considerable amount of bloody fluid. The cecum and the ascending colon contain much tarry material. The transverse and descending colon contain numerous firm scybalae.

The lesion described above in the small intestine extends to within six feet of the ileo-cecal valve. The lower six feet of the small intestine, the cecum, and the large intestine, including the rectum, are normal.

Peyer's patches and solitary lymph nodes are unaffected.

Brain.—Dura normal. Pia presents in places patches of opacity over the cerebral convexity, otherwise it is normal. Brain substance anemic. In the posterior pole of the left occipital lobe, there is an area of 3×5 cm., which is composed of softened brain substance. No yellowish color is noted.

Cortex over this area is extremely firm. Vessels at the base are normal. Lateral ventricles are normal.

Anatomical diagnosis.—Infective Endocarditis (Aortic); Mural Thrombus (R. auricle); Chr. Endocarditis (Aortic); Infective Aortitis of Sinus of Valsalva, with aneurysmal dilatation; Dilatation of R. Heart; Infarcts of Lung; Lobular Pneumonia (Discrete and Diffuse); Ac. Bronchitis; Sero-fibrinous Pleurisy and Pericarditis; Cirrhosis of Liver; Infarct of Spleen; Chr. Interstitial Nephritis; Thrombus of Common and Left External and Internal Iliacs; Encephalomalacia, Embolic, Infective (L. Occipital lobe); Ac. Hemorrhagic and Suppurative Enteritis.

Smears made at autopsy from the aortic vegetations showed very numerous slender threads, varying in length and thickness—some with bulbous ends, others tapering. No distinct branching. Decolorized by Gram's method. No metachromatic granules. No other bacteria seen.

Streaks and plates were made with glycerin and ascitic agar. The organism was not isolated in pure culture, transplants showing always an admixture of small Gram-positive bacilli and streptococci.

A typical streptococcus was obtained in pure culture from the pleural exudate, liver, spleen, and kidney. An attempt to cultivate the iliac thrombus yielded only a few contaminating colonies. No bacteria were found in smears from the thrombus, or from the softened area in the brain. Smears from the intestinal wall showed the usual polymorphous intestinal flora.

HISTOLOGICAL EXAMINATION.

The tissue was fixed in Orth's fluid, and imbedded in celloidin; for the demonstration of the bacteria in the tissues, prolonged staining with Löffler's alkaline methylene-blue was found satisfactory.

Aortic valves.—The aortic vegetations are composed principally of a dense, almost structureless tissue, which in places only has an indefinite fibrillar or laminated structure. Near the surface, but found also at a considerable depth within the substance of the valve, are irregular purplish clumps, stained more intensely at their periphery. Many of these have a fairly well-defined radial arrangement, distinguishable even with the low-power objective. With the immersion lens, in sections stained with Löffler's methylene-blue, these clumps are seen to consist of dense masses of the bacilli, to be described below, which form tangled masses or colonies, but at the periphery of the clump, evince a distinct tendency to arrange themselves in sheaves or parallel to one another.

The morphology of the individuals is identical with that of the micro-organisms found in the smears from the vegetations, and in the cultures from the blood during

life; the bulbous, irregularly stained filaments are readily made out. A few very long, wavy, distinctly branched forms are found, as well as numerous deeply stained coccoid bodies.

In one of the sections, there were to be seen scattered oval or polyhedral cells of large size, some with double nuclei, which suggested osteoblastic cells in appearance, although no typical bone trabeculae were present.

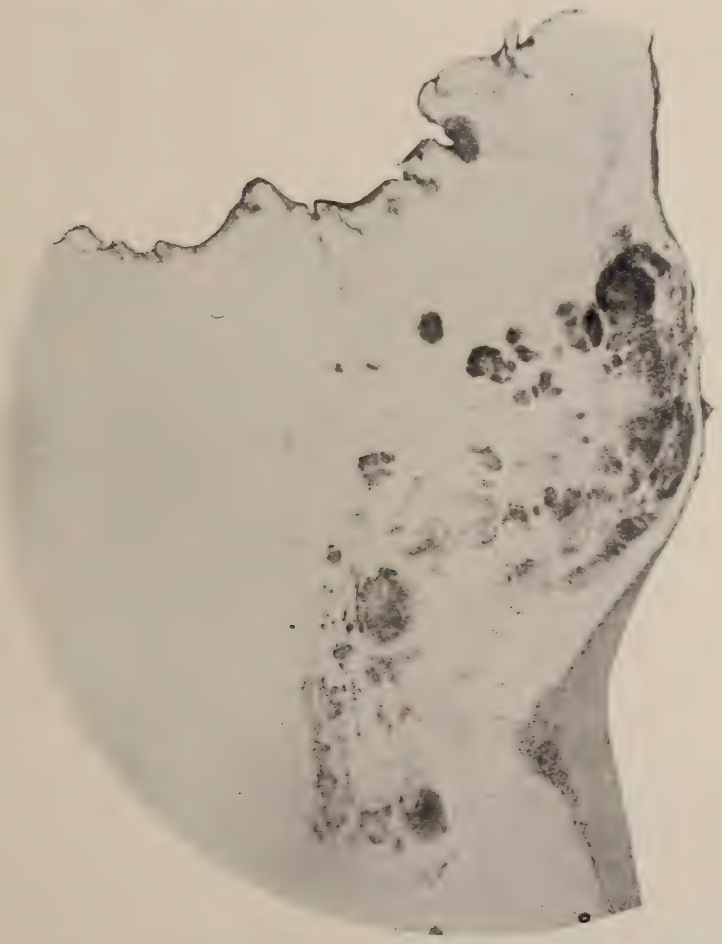

Fig. 2.—Section through aortic vegetation (low-power, Löffler methylene blue). Numerous colonies of filamentous bacilli within the depths of the vegetations.

Auricular wall and thrombus.—The thrombus is composed of red blood cells, leucocytes, chiefly polynuclears, granular, and reticulated fibrin. There is no evidence of organization. Sections stained with methylene-blue fail to show bacteria.

The myocardium, especially the papillary musculature is edematous and infil-

trated with leucocytes. The individual fibers are swollen, their striations largely obliterated. The smaller blood-vessels show thickening of the muscular coats, and proliferation of the intima.

Iliac artery.—Sections taken through the wall of the vessel at the seat of the thrombus, show the lesions of an acute arteritis, involving all the coats. The endothelium is exfoliated. The internal muscular coat, especially its middle portion, is infiltrated with polynuclear leucocytes, many of them pycnotic and fragmented. The external muscular coat, and adventitia, are thickened, edematous, and infiltrated with leucocytes. The vasae vasorum are sclerotic, and there is marked perivascular round-cell infiltration. The thrombus is composed of blood-cells and hyaline fibrin, and shows no organization.

Bacteria could not be definitely demonstrated, either in the wall of the vessel, or in the thrombus.

Pulmonary artery.—Sections through thrombosed portion: Clot shows organization at the periphery. Endothelium is swollen. The mesarterial coat is not changed, but the adventitia is thickened, and its vessels congested. The surrounding alveoli are filled with extravasated blood.

Lung.—In sections through the hemorrhagic infarcts, the alveoli are found packed with red corpuscles. The alveolar cells are swollen, many of them exfoliated. The walls of the alveoli are not destroyed, but in places show considerable recent connective tissue formation with sprouts of epithelioid cells projecting into the alveolar cells. In a section passing through a thrombosed vessel there is seen a partially organized clot, the center of which is puriform, consisting almost wholly of polynuclear leucocytes, more or less disintegrated. No bacteria are seen within the thrombus in methylene-blue preparations. Scattered through the alveoli are isolated pairs or chains of cocci, with an occasional plump bacillus. No micro-organisms of the type isolated from the blood and present within the heart valves can be found.

Sections through the consolidated portion of left lower lobe, present the ordinary lesions of a lobar pneumonia in the stage of red hepatization.

Liver.—The lesions found in this organ are simply those of extreme passive congestion. The liver cells, in the central portion of the lobules, are shrunken, their cytoplasm is granular and eosinophilic. Their nuclei lie at the periphery of the cells, are small, and stain diffusely. Many of the liver cells contain blood pigment. The peripheral portions of the lobules in places are the seat of fatty infiltration. Some of the cells exhibit cytolytic changes, such as have been described by Oertel,[1] namely. solution of the hyaloplasm, with preservation of the reticulum and nuclear membrane, The hepatic capillaries are much dilated, crowded with red blood cells and leucocytes. The central veins are engorged with blood.

No bacteria are found in the sections, although cultures made at the necropsy yielded a pure growth of streptococcus.

Spleen.—Sections were made through the yellowish infarct and adjacent splenic tissue. The infarcted area consists of a necrotic mass staining intensely with eosin, and under high magnification is seen to be composed largely of disintegrated red blood cells. Scattered through it are lymphoid cells and pycnotic nuclei. The margin of the infarct is formed by a cellular granulation tissue, in which are irregular epithelioid cells or fibroblasts, arranged with their long axis parallel to the long diameter of the infarct, lymphoid cells, new-formed capillaries, and abundant granular pigment, chiefly within larger phagocytes.

The splenic tissue itself shows the changes of chronic passive congestion. The

blood sinuses are thickly packed with red corpuscles; there are many large cells filled with granular blood pigment; the endothelial cells of the sinuses appear proliferated. The Malpighian follicles are small. The trabeculae are thickened and show hyaline degeneration.

No bacteria are found in sections, either within the infarct or at its margin, or in the splenic tissue. Cultures made at autopsy, however, yielded a pure growth of *Strept. pyogenes.*

Kidneys.—The epithelium of the tubuli contorti is swollen, irregular and granular. The lumina contain granular detritus. The tubes of Henle and the tubuli recti are in many places filled with casts. There are a number of areas of sub-capsular interstitial inflammation. The glomeruli appear normal.

The large yellowish area noted in the gross corresponds to a necrotic infarct composed of hyaline tubules, vessels, and connective tissue. The vessels show an advanced sclerosis, thickening, and hyaline transformation of the media and adventitia.

The bright yellow color, which was a striking feature in the fresh specimen, appears to be due to the deposition of golden or yellowish-brown pigment granules (hemosiderin). In some places, especially in the cellular connective tissue at the margin of the infarct, this pigment deposit is very abundant indeed.

No bacteria are found. A pure growth of streptococci was, however, obtained at autopsy.

Adrenal.—Sections present a normal structure. The capillaries are perhaps somewhat congested.

Pancreas.—Sections of the pancreas show no noteworthy lesion. The blood-vessels everywhere are markedly congested. The acini and islands of Langerhans are entirely normal.

Small intestine.—In sections taken through that portion of the gut which in the gross showed a necrotic inflammation, the mucosa is completely exfoliated, only a few deeper acini persisting. The internal surface of the gut is formed by a thick layer of exudate, consisting of polynuclears, granular and hyaline fibrin, red blood cells, and large bacterial colonies. The submucosa is very edematous, the blood-vessels and capillaries intensely injected. The fibers of the muscularis mucosae in many places are torn apart and the purulent exudate extends into the submucous tissue. The muscular layers and peritoneum are not affected, save that the blood-vessels are intensely engorged with blood, and the intermuscular connective tissue appears edematous.

Brain.—Sections from the left occipital lobe, through area of softening: Softened tissue is composed of an edematous meshwork of neuroglia fibers, in which are new-formed capillaries, and many large phagocytic cells, containing red corpuscles and blood pigment. Free unaltered red cells are found in considerable numbers. The adjacent brain tissue shows gliomatous proliferation, and there is a productive inflammation of the overlying pia-mater.

No bacteria are found in sections stained with methylene-blue. The histological picture is therefore that of a fairly recent thrombotic softening undergoing repair.

BACTERIOLOGICAL.

Reference has been made to the bacteriological examination undertaken at the necropsy, and to our failure to obtain in pure culture the micro-organism which was present in the aortic vegeta-

tions. The description of the cultural characters is therefore based upon a study of the micro-organism obtained upon two occasions from the circulating blood during life.

On November 15, a fourth blood culture was taken, the previous ones having been sterile. About 6 c.c. were withdrawn from a vein and distributed into three agar plates and one broth flask. After three days, about 25 deep and superficial colonies developed on each plate. The flask showed only a few discrete whitish colonies at the bottom, which proved to be *Staph. albus*.

Subcultures were made from the plate colonies on human-blood-agar slants, from which further transplants were made and the morphological and cultural features determined. A fifth blood culture, four days later, yielded a pure growth of an organism identical in all respects with that obtained from the previous culture. Each plate showed, after 48 hours, from five to ten deep and superficial colonies; the flask remained sterile.

Morphology.—The most striking feature of the micro-organism is its pleomorphism. Pronounced variation in the length, thickness, and conformation of the individuals is constantly present, irrespective of the particular medium or stage of growth. Measurements therefore are of little value, since in every culture examined there are seen gradations from short bacillary forms, ranging from 2–6 μ in length up to long filaments crossing the entire field of the immersion lens, and measuring 100 μ or more in length. In hanging-drop preparations the long, threadlike forms usually exhibit more or less definite segmentation, and appear to consist of a series of individuals joined end to end. Such segmentation, however, is not always to be distinguished, even in fresh preparations, and in stained smears an interruption in the continuity of the filaments can rarely be made out.

Variation in thickness of the individuals is also well marked, slender forms about the thickness of a tubercle bacillus perhaps predominating. The caliber is rarely uniform; frequently there is a bulbous or clublike swelling at one or both extremeties, and occasionally fusiform enlargements at one or more points along the course of the filaments. The shortest forms are usually straight, the longer ones slightly curved, and the filaments often wavy.

Branching has been observed in hanging-drop preparations from

broth cultures, in smears from fluid and semi-solid media (Hiss-tube medium). The precise nature of this branching is difficult to study because of the tenuous character of the micro-organism, and the fact that it grows extremely slowly or not at all at room temperature. We are nevertheless of the opinion, from a careful scrutiny of hanging-drop preparations, that *true branching*, in the sense of a lateral budding or terminal dichotomization does *not* occur, but that the forked and divisional forms are produced by a process of *pseudo-ramification*. By this is meant that two adjacent segments becoming bent upon themselves, the sheath or membrane is ruptured at the point of juncture, and the continued growth of one segment in the line of the original axis gives rise to a forked or branched appearance.

Branches are invariably given off at the juncture of two segments, and there is usually to be made out a slight gap between the main stem and the branch. Forked forms are met with, in which the two secondary segments lie at equal angles to the primary axis. In other cases, the branch is given off at a right angle.

The significance of the distinction between true and false branching in attempting to establish the identity of the micro-organism, will be discussed later.

Staining reactions.—The organism stains readily, but not intensely, with all the usual anilin dyes. Prolonged staining with Löffler's alkaline methylene-blue or dilute carbol fuchsin has given the best pictures. In some preparations, the presence of metachromatic granules, usually two to each rod, could be readily demonstrated. Their location varied—in some individuals the granules were situated at the poles; in others, one or both were found in the central portion of the rod. The long filamentous forms frequently showed a considerable number of these granules. In later generations, metachromatic granules, though occasionally present, were not often seen.

Very interesting pictures were obtained in films fixed with methyl-alcohol, and stained by Giemsa's method. In these, the rods and filaments take a rather pale-blue stain; at irregular intervals are brilliantly stained purple granules, spherical, oval, or occasionally in the shape of short cylindrical masses with rounded ends. The terminal bulbs may be pale; frequently they contain a single purple granule, and occasionally the entire bulbous enlargement takes an intense purplish stain. The purple staining substance is not invariably distributed in the form of discrete granules or rods, but the longer filaments may take a diffuse purple color over a considerable portion of their length. Frequently, the micro-organism appears to be slightly swollen in its deeper-staining portions.

The organism is readily and completely decolorized by alcohol in less than thirty seconds, when stained according to Gram's method. It is not acid fast.

Involution forms.— A variety of irregular, swollen, and beaded forms which stain poorly, are found in old cultures. In stained smears from a dry 20-day-old agar culture, numerous very long filamentous forms are seen, many of them showing a terminal deeply staining bulbous swelling. Whether these appearances are to be considered as involution forms associated with unfavorable conditions of growth is not clear; it is to be noted that similar forms were found in the smears made from the cardiac vegetations. In fluid media or in recent agar cultures on moist media, on the contrary, very long forms with terminal bulbous enlargements are less frequently seen.

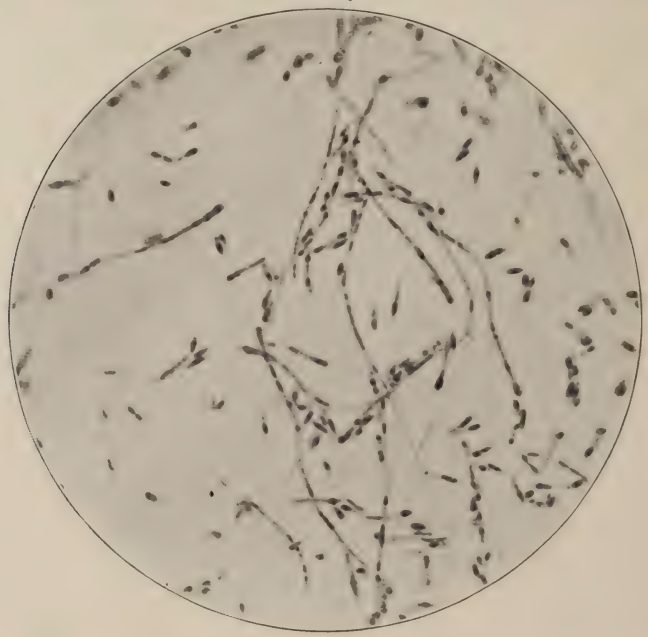

FIG. 3.—(Eleven-day agar culture, Giemsa stain, ×1,000 diam.) Filamentous and bacillary forms with deeply stained chromatin substance.

It is possible that the terminal swelling may be a reaction to the mechanical resistance of the firm tissues or solid media—an explanation of which has been offered and generally accepted for the origin of the clublike terminal swellings of the actinomyces within the tissues (Lachner-Sandoval,[2] Petruschky,[3] Wright[4]). Some of the short, greatly swollen segments resemble budding yeast cells in their appearance, and must undoubtedly be regarded as degeneration forms.

Capsules.—Films from recent agar cultures stained by Wright's method show the individual rods and filaments to be enveloped by an unstained clear space, which is bounded by a fine but distinctly stained line. The breadth of this clear space varies, but it is relatively about as large as the capsule of the *Streptococcus mucosus.* Such a capsule could not be constantly demonstrated, but appeared to be present whenever the cultures possessed a sticky or mucoid character. That the appearance described indicates a true capsule and not an artefact, is evidenced by the presence of numerous empty capsules.

Films stained by Buerger's method present a similar picture, with, however, certain differences. The filaments themselves appear thicker than with Wright's stain, the clear spaces relatively narrower, and the limiting membrane, if we may use the term, less sharply defined.

The Hiss copper sulphate stain and the Welch stain gave similar, but less instructive, pictures.

Motility.—The micro-organisms are absolutely non-motile.

Spore formation.—In all cultures, but more abundantly in older growths, there

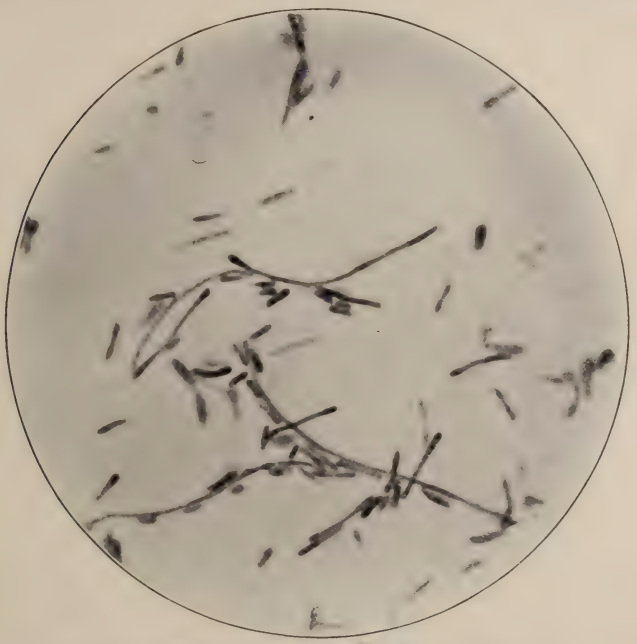

Fig. 4.—Four-day Löffler blood-serum culture. Dilute carbol fuchsin. ×1,000.

have been noted free oval or coccoid bodies whose appearance might suggest spore-formation. We are, however, disinclined to interpret them as such, but to consider them rather, as the granular remains of disintegrating bacillary forms, since they do not respond to the ordinary spore stain, and are readily decolorized by acid and alcohol.

The globular and spheroidal masses which take a purple or nuclear stain in Giemsa preparations, we consider to represent chromatin substance rather than endospores.

An attempt was made to determine the thermal death-point of the micro-organism by suspending a recent agar culture in broth, heating to various degrees of temperature in capillary tubes, and re-transplanting on agar slants. It was, however, difficult to obtain a growth from control suspensions. The experiment was therefore unsuccessful.

Old agar cultures showing extreme thread formation were repeatedly examined

for evidences of aerial spores. Chains of terminal gonidia, such as are invariably present in old cultures of streptothricae, were never observed.

Cultural characters.—The organism is an obligate-aerobe. Tubes incubated under strict anaerobic conditions (Novy apparatus) failed to show appreciable growth after 11 days, but grew readily when the tubes were subsequently incubated aerobically. Growth at room temperature takes place very slowly or not at all.

Broth.—The appearance varies slightly with different cultures. There is sometimes to be noted a flocculent cloud after 24 hours, near the surface of the tube, which

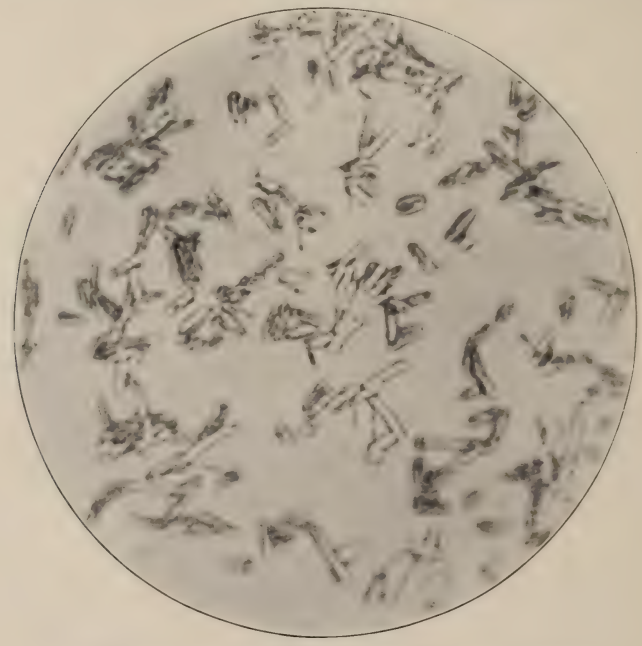

FIG. 5.—Recent agar culture. Wright's stain. ×1,000. Capsules.

gradually settles to the bottom. After two or three days, there is seen at the bottom of the tube a delicate scaly or sandy sediment; on shaking, coarser opaque whitish granules are seen in the flocculent deposit. After prolonged transplantation the appearance of the cultures in broth has changed somewhat. After 24 hours, there appears a diffuse turbidity of the broth, with the deposition of a fine, scaly, whitish sediment along the sides and at the bottom of the tube. Further growth does not alter the appearance noticeably, save that the deposit becomes more abundant, and sediments completely as a whitish scaly deposit, leaving the supernatant broth clear. No pellicle is formed. There is no odor to the cultures.

Broth cultures tested for indol after 11 days, gave a fairly marked positive reaction.

Gelatin.—No growth is observed at room temperature, even after several weeks. There is no peptonization, as shown by growing at 37° and then solidifying.

Agar slants.—The organism grows readily on plain agar. After 24 hours, if a large amount has been transplanted, there appears a diffuse confluent, rather sticky,

grayish-white growth; as the medium becomes dryer, the growth acquires a cohesive or tenacious character, so that it adheres rather firmly to the loop. As the agar dries, the surface of the growth shows a glistening, satin-like luster.

When a small amount of growth has been transplanted, discrete colonies are formed which gradually assume the appearance illustrated in Fig. 6. They attain a size not exceeding 1–2 mm., are yellowish-white in color, with a slightly raised, dome-shaped central knob or projection, which appears stippled and brownish by transmitted light. The peripheral portion of the colony is flat, the surface uneven or wrinkled, the margin irregular, finely serrated and raised slightly above the surface of the agar. The colonies have considerable cohesion, but do not adhere firmly to the medium.

Viability.—Transplants were readily obtained from agar cultures after two weeks

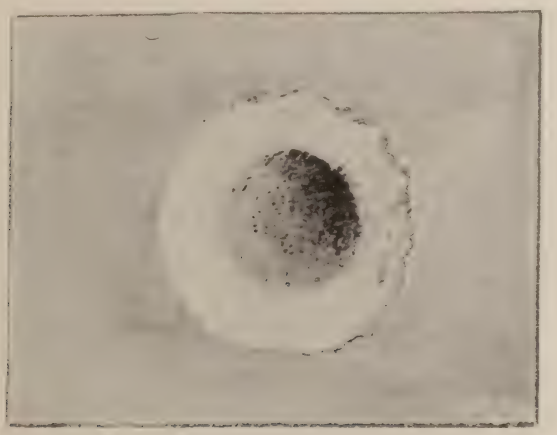

Fig. 6.—Eleven-day agar culture: single colony.

growth at incubator temperature, without special precautions against drying. No detailed study of the resistance of the micro-organism to drying was made.

Glycerin-agar slants.—The appearance of the growth is identical with that on plain agar. The organism, however, tends to die out more quickly on this medium.

Blood-agar slants.—(Human blood streaked on plain agar.) Abundant confluent growth, without striking characters.

Blood-agar plates.—Upon the four plates, obtained by blood-culture (1–2 c.c. of blood to 10 c.c. of agar), the colonies appeared slowly, becoming visible only after 48–72 hours. The superficial colonies were 1–2 mm. in diameter, slightly raised above the surface of the medium, pearly white in color, tenacious in consistency. There was no hemolysis or discoloration of the medium. The maximum size (about 2 mm.) was attained after four days.

Litmus milk.—No acid production or visible change in milk cultures. Smears made after three or four days show only poorly staining, apparently degenerated forms. Milk does not, therefore, appear to be a favorable medium.

Potato.—No visible growth.

Hiss-tube medium (gelatin-agar + 1 per cent dextrose).—Grows readily along stab as whitish granular streak, somewhat more diffused near the surface. There is no clouding of the media, and no gas production.

Löffler coagulated blood serum.—Growth is not always successful. When it occurs, it appears as a moist, grayish-white confluent surface growth, with a flocculent precipitate in the condensation water. There is no liquefaction of the serum.

Fermentation reactions.—Cultures on sugar-free broth + 1 per cent saccharose, dextrose, lactose, and mannit. There is no growth in the closed arm of the tubes and no gas production. The appearance of the growth in the bulb and bend of the tube is identical with that described in ordinary broth.

Cultures on carbohydrate serum-water media. In *dextrose-serum-water*, there is acid production, visible after 48 hours and gradually increasing. The acid produced is not sufficient to cause coagulation of the serum. Acid is also formed in *maltose-serum-water*, but is produced more slowly and in less amount than in dextrose. Saccharose, lactose, mannit, dextrin, and inulin are not fermented.

Pathogenicity.—The micro-organism is non-pathogenic for guinea-pigs and rabbits.

A guinea-pig inoculated (November 19, with whole 24-hour blood-agar culture remained alive and well until March 8. It was then chloroformed. No visible lesions were found.

March 4. Three guinea-pigs were inoculated intraperitoneally with saline suspension of 48-hour agar cultures. No local reaction, or evidences of illness. Pig No 1, inoculated with 5 c.c. of the suspension, was killed after nine days. Careful examination showed no lesions. The remaining pigs have shown no signs of illness, and are still alive.

A rabbit inoculated intraperitoneally on March 4 has remained alive and well (June 13, 1907).

GENERAL CONSIDERATIONS.

The systematic classification of the micro-organism here described presents considerable difficulty, and we may say at the outset that we have not encountered in the literature any bacterium which closely resembles the one here studied. If we review its salient characters, we find: (1) pronounced pleomorphism; (2) tendency to the formation of long filaments, usually with terminal bulbous swellings; (3) the presence of a readily demonstrable capsule; (4) false branching, in fluid or semi-solid media; (5) a distinct disposition toward a radial arrangement of the individuals in the tissues; (6) abundant growth upon the usual laboratory media. Negative characteristics, which are of importance in determining the systematic relationships of the micro-organism are: (1) absence of motility; (2) the probable absence of spores; (3) its failure to retain the Gram stain; (4) its strict aerobic growth; (5) its lack of pathogenicity for guinea-pigs and rabbits.

The three groups of bacteria which would come under consideration in discussing the questions of identity are: (1) the pleomorphic bacteria—a loose term taken to include the various acid-fast bacilli, the non-acid fast diphtheria group, the bacillus of glanders, the proteus group, the anaerobic *B. necroseos*, *B. fusiformis*, and other less well-known varieties; (2) the capsulated bacilli, including the Friedländer and aerogenes groups, and (3) the trichomycetes, if we adopt the term which Petruschky[3] has used to include the various Actinomyces, Streptothricae, Cladothricae, and Leptothricae. With each of these three groups, the micro-organism which we have studied presents certain obvious affinities, and even more obvious points of difference. The extreme pleomorphism which it exhibits, and especially the constant occurrence of long filaments, differentiates it from any of the well-known members of the first group, and serves to exclude it without further comparison from the second group of capsule bacteria. Many bacteria may display a certain degree of pleomorphism, especially under unfavorable cultural conditions. With the micro-organism studied, however, this variation in the size and configuration of the individuals is a constant and distinctive character, and on morphological grounds alone is sufficient to differentiate it, for example, from the diphtheria and pseudo-diphtheria group. To the other pleomorphic bacteria above mentioned it has even a fainter resemblance. If we attempt, therefore, for purely descriptive purposes, to group this micro-organism with the pleomorphic bacteria, it must be with the reservation that it departs in many special features from all the known varieties commonly included in the term.

The tendency of this micro-organism to grow in long filamentous forms, to develop terminal clublike swellings, and to exhibit a fairly well-marked radial arrangement in the tissues, suggests certain affinities to the group of *Streptothricae* and *Actinomyces*. These resemblances, nevertheless, are superficial. There are fundamentally important points of difference. The organism does not retain the Gram stain; it does not form a true mycelium, with terminal aerial spore chains; branching forms are met with only exceptionally, and true branching apparently does not occur. Other important differential characters are the rapid growth on the usual laboratory media,

the presence of capsules, the absence of pigment production, and the character of the colonies.

The relationship of our micro-organism to the little studied group of *Leptothricae* may be very briefly considered. This group is characterized by the formation of long rigid filaments, of uniform caliber, rarely curved or wavy, and exhibiting neither branching, segmentation, nor aerial spore formation. Their mode of development is little understood, since artificial cultures have been successful in but two instances.[5] Besides *Leptothrix buccalis*, found as a normal inhabitant of the mouth, and the closely related or identical species associated with "Mycosis of the pharynx and tonsils," and the well-known Boas-Oppler bacillus, Leptothricae have been found but a few times in association with pathological processes: in the urine of a tabetic,[5] in a case of vaginitis,[6] and in a gangrenous phlegmon of the lower jaw.[7]

In 1896 Flexner[8] discovered, in a case of puerperal endometritis in a rabbit, a filamentous bacillus which he cultivated aerobically upon sterile organs, but which did not grow upon the usual laboratory media. To this organism he gave the name *B. pyogenes filiformis*, and grouped it provisionally as a Leptothrix. In experimental lesions in rabbits, he obtained a radial or sheaflike arrangement of the filaments which resembles rather strikingly the arrangement the micro-organisms in the colonies within the aortic vegetations, in our case.

Galli-Vallerio,[9] in smears and broth cultures from subcutaneous tuberculous abscesses in man, found, in conjunction with the tubercle bacillus, a long threadlike organism which decolorized by Gram's method and exhibited false branching. Subcultures were unsuccessful and inoculation experiments negative. A detailed description of this bacterium, classed by the author as a Leptothrix, is not given.

As regards the group relationship of our bacterium to the Leptothricae, analogies are found only in its filamentous character and in its irregular staining. The occurrence of bulbous terminal enlargements, of false branching, of bent and wavy forms differing from the rigid, straight threads of the leptothrix, differentiate it morphologically from the hitherto described members of the group. Its

ready growth on various media seems to us also a differential point of importance notwithstanding the unconfirmed report of Arustamoff,[5] as to the cultivation of his two species.

Without further analysis, therefore, we are led to reject the designation of leptothrix for the micro-organism which we have described.

Under the genus *Cladothrix*, which by Kruse[10] is grouped with the Bacteriaceae, by Petruschky[3] with the Hyphomycetes (sub-order Trichomycetes), three species have been described: the *C. dichotoma* (Cohn), the *C. ochracea* (Winogradsky), and the *C. intricata* (Russell). These species are obtained from water, are non-pathogenic, and do not grow readily upon laboratory media.

A number of authors, however, have described under the term "Cladothrix" micro-organisms found in necrotic or granulomatous lesions in man or animals, which upon subsequent examination by more recent workers have been included under the Streptothricae. Thus the filamentous organism described by Nocard[11] as *Cladothrix farcinica* (found in the *farcin de bœuf* of Guadeloupe) was later shown by Sauvageau and Radais[12] to exhibit true ramification and to be in all probability a true Streptothrix. So also, the *Cladothrix asteroides* found by Eppinger[13] in a brain abscess and in the bronchial and supra-clavicular lymph-nodes, is stated by Kruse[10] to show true branching and to belong rightfully to the Streptothrix group. Rossi-Doria,[14] working with the same strain, came to a similar conclusion. Petruschky also says that "the resemblance to the Streptothricae is very close." In spite of Eppinger's direct assertion as to the occurrence of false branching, his illustrations certainly do not convey that impression. Whatever may be the proper classification for Eppinger's organism, it is readily differentiated from the one which we have studied by its warty growth on solid media, the production of ochre-colored pigment on old cultures, the reddish growth on potato with the formation of aerial spores and the behavior to Gram's stain.

The filamentous micro-organism isolated by Rosenbach[15] from erysipeloid infections (erythema exudativum multiforme) and described by him as a Cladothrix is classed by Kruse, Lachner-Sandoval, Rossi-Doria, and others as a Streptothrix, and shows no resemblances to the micro-organism here described.

In 1888, Naunyn[16] reported finding in the endocarditic vegetations from a case of chorea, and in the foci of hemorrhagic encephalitis, a branching micro-organism which was identified by Zopf as a Cladothrix or Leptothrix. Unfortunately, the original publication of Naunyn is not accessible, and the abstract in *Baumgarten's Jahresberichten* is too meager to permit of a comparison between this organism and ours. This is the only case, however, so far as we can ascertain, in which bacteria of this group at all resembling the one found in our case have been encountered in endocarditic processes.

The branching filamentous organism described by Hesse[17] in 1892 under the name *Cladothrix liquefaciens*, should be grouped with the Streptothricae, so also an organism isolated by Bornhaupt[18] from a case of pseudo-actinomycosis in man, and identified by him with the "*Cladothrix liquefaciens*" of Hesse.

Rullman's[19] organism, probably a Streptothrix, isolated by him from the sputum in a case of pulmonary disease, shows morphological similarities, but differs very distinctly in its pathogenic and cultural characters.

In spite of the fact that no pathogenic organisms belonging beyond question to the group Cladothrix have as yet been recorded, it seems to us that the micro-organism found in our case answers to the rather ill-defined characters of this group sufficiently to consider its classification, tentatively at least, as a Cladothrix. It fully meets the salient characters of the group, if we accept the classification of Petruschky in Kolle and Wasserman's *Handbuch*, in so far as it exhibits a "false branching (lateral rupture of the sheath with continuation of longitudinal growth in another direction), rapid fragmentation and in consequence a bacillary character in older cultures." On the other hand, the presence of distinct capsules, and the more important fact that no micro-organism belonging with absolute certainty to the Cladothricae have as yet been found to possess pathogenic significance makes us hesitate to identify the micro-organism which we have described with this group.

Moreover, the limits of this class of trichomycetes are not sharply defined. Although Petruschky somewhat arbitrarily groups the Cladothrix under the Hyphomycetes, and sharply separated them from the higher pleomorphic bacteria, he admits that the phylogenetic

relation is a close one. On the other hand, Neumann and Lehmann[20] include the pleomorphic bacteria with the Hyphomycetes. Until a rigid and universally acceptable classification is made, we prefer not to attempt to assign a definite place to the organism studied by us, but to recognize its relationships to the pleomorphic bacteria, on the one hand, and to the Cladothrix group on the other.

To sum up, the micro-organism shows the following features: Long, slender, non-motile bacilli, showing marked pleomorphism; filamentous forms, with distinct segmentation and pseudo-ramification; irregular staining, with the presence of metachromatic granules; decolorization by mineral acids and failure to retain the Gram's stain; strict aerobic growth on all the usual laboratory media save potato; no pathogenicity for guinea-pigs and rabbits. The organism was obtained from the blood on two successive occasions, and an organism presenting identical morphology and staining reactions was present in great numbers in smears and sections of the aortic vegetations, but was not isolated in a pure culture. In the sections of the aortic vegetations, the colonies exhibited a definite and striking radial arrangement of the individual organisms.

We consider the causative relationship of this micro-organism to the production of the vegetative endocarditis to be beyond question, because of its presence in great numbers within the depths of the thrombotic deposits, and because of its presence in pure culture in the circulating blood during life. The general streptococcus infection found at autopsy, we believe to be a terminal condition, since the streptococci were not found during life in spite of repeated blood cultures, and were not present in the depths of the vegetations.

In conclusion we wish to express our indebtedness to Dr. Norris, director of the laboratory, for his advice and assistance, and to Dr. Jaches of the Cornell University Medical School, and Mr. E. Glueck, for the photographic illustrations.

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AN IMPROVED AND RAPID TEST FOR INDOL IN BROTH CULTURES AND FOR THE PRESENCE OF THIS SUBSTANCE IN MEAT-SUGAR-FREE BROTH.*

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In my previous paper read before the Pathological Society of Philadelphia, March 14, 1907, entitled "Contributions to the Differentiation of *B. coli communis* from Allied Species," we had the opportunity of bringing out some points and reactions as well as some tests characteristic of *B. coli* by means of which this organism could be differentiated from the rest of the Colon group. When I say, from the Colon group, I wish to state again that, based upon our results and observations, *B. coli communis* presents some constant biological features peculiar to itself, by means of which the differentiation can be easily accomplished. As a matter of convenience, these reactions were named "Test 1," "Test 2," and "Test 3," respectively.

Test 1 is a negative test. When about $\frac{1}{4}$ c.c. of sterile dextrose broth is boiled for a few minutes in about 5 c.c. of a 10 per cent sodium hydroxide solution, a light yellow canary color is produced. Similar treatment of a 48-hour-old culture of *B. Coli* produces exactly the same result, whereas with allied species a pinkish coloration is imparted to the liquid on standing from 5 to 15 minutes.

Test 2 consists in a bright purple or pinkish coloration produced by *B. coli* when about 1 c.c. of a 10 per cent sodium hydroxide solution and about 1 c.c. of a 50 per cent sulphuric acid solution are added to the culture; cultures of the saccharolytic group produce no such reaction. A study of the nature of this reaction has proven it to be very closely connected with indol, or at least with some derivative of indican.

Test 3 consists in the inability of *B. coli* to exhaust the sugar in a 1 per cent dextrose broth, the action on this substance ceasing after 48 hours at 37° C., and sometimes as early as 18 hours, while allied

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species, regarded as the Colon group, go on uninterruptedly until the sugar is completely exhausted. In view of this peculiarity I believe it to be logical to substitute the name of "saccharolytic group" for these Colon-like organisms. I deem it unnecessary to go into details of the test for determining partial exhaustion of the sugar by *B. coli* and complete exhaustion of this substance by the saccharolytic group. This subject was thoroughly considered in the previous paper, and it suffices here to say that the test can be determined by the polariscope, or more practically by Fehling's Solution, as in testing diabetic urine.

It is not our purpose to go deeply into the exact chemical nature of the reactions, but more especially to determine the following points in regard to them:

1. *Relation of Test 2 to B. coli and the saccharolytic group.*—After exhaustive observations upon a number of cultures of true *B. coli* and those considered to belong to the saccharolytic group, I believe that my results demonstrate that Test 2 is characteristic of the former, and that any culture which does not show this reaction in spite of other biological characters, should be discarded as a true Colon and classified among the saccharolytes.

2. *Time required to obtain a positive reaction.*—To my satisfaction, it was found that Test 2 does not require the long wait of two to eight days required by the sulphuric acid and potassium nitrite test, but only one, or at the most two days of incubation at 37° C. In fact I have obtained this reaction on *B. coli* after six hours only, or in a shorter time, between four and five hours at 37° C., as soon as the slightest cloudiness of the medium was observed; this was never the case with the sulphuric acid and potassium nitrite test applied for comparison.

3. *Relation of Test 2 to the sulphuric acid and potassium nitrite test.*—As a matter of routine in studying a great number of cultures isolated from different sources and compared with a *B. coli* culture as control, both tests were applied simultaneously to the same culture, and the result corresponded very closely to the test for indol. In a few instances, however, Test 2 (sodium hydroxide and sulphuric acid) was positive, while the test for indol (sulphuric acid and potassium nitrite) remained negative, not because such cultures did not

produce indol, as the same thing was observed in the *B. coli* cultures, but because the test with sodium hydroxide and sulphuric acid was found to be more delicate and precise than the test with sulphuric acid and potassium nitrite, as determined by the following observation:

4. *Differences in Test 2 and indol test with different strength solutions of indol.*—Experiments with different strength solutions of indol crystals in distilled water were made and tested simultaneously by both tests. The sulphuric acid and potassium nitrite test gives a salmon-amber color, somewhat resembling the normal color of broth, while the sodium hydroxide and sulphuric acid give a bright purple-pinkish color decidedly more distinct than the sulphuric acid and potassium nitrite test.

5. *Delicacy of both tests.*—The sulphuric acid and potassium nitrite test was positive to the dilution of 1 : 1,000,000—that is, when the test was applied with all precautions and concentrated in forms of rings; it was almost indistinct in 1 : 800,000 when tested otherwise. Test 2 (sodium hydroxide and sulphuric acid) was found to be positive in 1 : 1,400,000, regardless of any precaution in making the test since this does not depend upon any concentration of the reactions but upon a diffuse general coloration of the medium. Further, to my satisfaction, by making the dilution with broth instead of distilled water, it was observed that the sodium hydroxide and sulphuric acid produced more or less destruction of the coloring matter of the medium, leaving an almost colorless broth upon which the reaction appears more pronouncedly, while the sulphuric acid and potassium nitrite produced no change in the color of the broth, which in some ways obscures the salmon-amber color of the reaction.

It was further noted that the color of the broth has an important bearing upon the sulphuric acid and potassium nitrite test, the darker the medium the less distinct being the reaction. Following this line of observation we found the test to be positive in some cases in the concentration of 1 : 500,000 only, and not beyond that point. Therefore for this reason, if for no other, Test 2 (sodium hydroxide and sulphuric acid) is preferable to sulphuric acid and potassium nitrite. Further it is not necessary to concentrate the reaction in the form of rings a method requiring a careful technic by no means always successful, but merely to add the sodium hydroxide and sul-

phuric acid without any special precaution. The coloration is a diffused bright purple-pink, of itself sufficiently distinct and characteristic.

Having determined by the above experiment that Test 2 (sodium hydroxide and sulphuric acid) bears a very close relation to the sulphuric acid and potassium nitrite test for indol, and having observed that this test is not only more delicate and in many ways superior and more easily performed, attention was next directed to determine if the meat-sugar-free broth made by the previous fermentation and exhaustion of the inosite in the meat juice during the incubation of 18 to 24 hours at 37° C., a method suggested by Smith and accepted by all bacteriologists, could be regarded as free from indol. It is stated that such preliminary fermentation by *B. coli* does not produce any perceptible amount of indol; however, in an effort to determine the correctness of both assertions, it was desirable to make some observations upon the subject. That indol is never produced in the presence of sugar is a well-known fact, but is it not possible that the amount of sugar present in the meat juice would be so small as to be easily exhausted by the *B. coli* in a few hours so that in the remaining time this organism would attack the proteid substances in the meat juice sufficiently to transform them into indol? Having determined that the sulphuric acid and potassium nitrite test for indol is not very delicate, and being in possession of Test 2 which showed itself to be more delicate and reliable, some experiments were conducted to determine the presence or absence of indol in meat-sugar-free broth.

Meat juice was tubed and sterilized in the autoclave at 20 pounds pressure for 20 minutes and a series of tubes inoculated with *B. coli* cultures (the amount inoculated was 1 drop of a 24-hour-old broth culture, this small amount being employed to avoid any possible error from the material transferred) and placed at 37° C. A number of tubes were tested by Test 2 after 2, 4, 6, 12, 18, 24, and 48 hours respectively. In some cases a positive reaction was obtained as early as after six hours' incubation. Most of the tubes showed a positive reaction after 12 hours, and this was more marked after 18 hours of incubation at 37° C.

Following the same line of experiments, the meat juice was tubed and without any preliminary sterilization, inoculated with *B. coli*

and incubated at 37° C. The test was applied as before, and the result was much the same. Further, with the idea that perhaps the subsequent sterilization would produce some changes in the indol occurring during the preliminary fermentation of the meat juice both experiments were repeated, but this time the test was applied after submitting some tubes to 100° C., and others to 20 to 30 pounds for 20 to 30 minutes. In both cases the heat was found to have had no effect on the reaction, as it was as typical and distinct as when no heat had been applied before performing the test, proving beyond any doubt that the subsequent sterilization, that is, the heat, has no effect on the reaction. This substantiated my experience in finding a positive reaction of indol in sterile broth control tubes, as well as in medium stored for laboratory use, and to this, no doubt, is due our recent literature on indol-positive typhoid strains.

As a matter of corroboration, and especially in order to determine in a more precise manner whether this substance was produced during the preliminary fermentation of the meat juice by *B. coli*, under exactly the same conditions the sulphuric acid and potassium nitrite indol test was applied in all the above experiments and the reactions were found to be negative after six hours. In one case only a very slight indol ring was obtained; in a few instances the reaction was concentrated in the form of rings after 12 to 18 hours and usually this was positive after 24 hours of incubation at 37° C.; this proves beyond a doubt the presence of indol sometimes in the meat-sugar-free broth. It is not true that all meat-sugar-free broth contains indol. In some experiments I was unable to detect this substance, due probably to an excess of acidity in the meat juice or to unfavorable conditions under which the preliminary fermentation was carried on, or to some inactivity of *B. coli* itself, which inhibited its action on the proteid substance, and under such circumstances it is a question whether even the sugar has been exhausted from the juice and whether such a broth can be regarded as free from this substance. It is a question, I believe, if this preliminary fermentation be desirable in order to exhaust the sugar in the meat. If so I would recommend the use of the saccharolytic group which rapidly attacks the sugar and produces no indol. Such cultures can be easily isolated from water and be used with advantage

instead of *B. coli;* I use for the present some of these cultures isolated from water producing 80 to 100 per cent of gas in 24 hours, which under the most delicate test have given negative indol reactions.

Before concluding, it is my desire to state that Test 2, if it be not a test for indol, can be properly regarded as something very closely related to it. Inasmuch as it is characteristic of *B. coli* it seems justifiable to use it in determining the identity of this organism, even though it may not be the same as the indol test.

In conclusion, I think the points to be emphasized from the results are:

First, the ease with which the test may be applied.

Second, the distinct and characteristic color.

Third, its applicability after an incubation of 24 hours, whereas by the ordinary indol test, a culture of eight days is recommended. (In this laboratory, 48 hours' incubation is given with the most satisfactory results.)

Fourth, the reaction does not have to be concentrated in the form of a ring.

Fifth, the rapidity with which a culture may be identified as a true *B. coli.*

Sixth, *B. coli* should be discarded as an agent for exhausting the sugar in broth, and one of the saccharolytic group used instead.

THE RELATIVE RATE OF GROWTH OF MILK BACTERIA IN RAW AND PASTEURIZED CLEAN MILK.*

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The significance of the relative rate of growth of various organisms in raw and heated milk has, during comparatively recent years, brought out a considerable mass of work the results of which vary widely, as one would expect, considering the difficulties in obtaining material, in technique, and in overcoming or accounting for the many variations, small in themselves, but of importance when dealing with so delicate a problem in a medium so readily influenced. Hence, one is not surprised to find Fokker,[1] Hesse,[2] Schottelius,[3] Kitasato,[4] Friedrich,[5] Heinemann,[6] and others maintaining a germicidal activity for fresh cow's milk, which activity is destroyed by heat, and equally positive assertions on the part of Basenau,[7] Heim,[8] Kleimmer,[9] Conn and Esten,[10] and others that such a power is not manifested by raw milk; while the intermediate school is represented by Moro-Graz,[11] Schenk,[12] Cozzolino,[13] etc., who would assign such a property to milk from some animals and not to that from others.

Much of this variation of opinion we believe to be due to the conditions under which experiments were performed, notably, the method of obtaining the milk. Unless drawn from the cow in a sterile condition it cannot be rendered so without alteration, to a greater or less extent, of those substances upon which the "life" of the milk depends, and while possible it is difficult, first, to find a cow giving sterile milk and, then, to draw it in a sterile condition. However, such experiments have been made and the results obtained by sowing pure cultures of various organisms in sterile, fresh milk would indicate that such milk does inhibit growth for some organisms more and for some less strongly.[14]

While such experiments are of great value they leave untouched, except by inference, a crucial point in the practical pasteurization and sterilization of milk for infant-feeding or for the general public which the large city milk dealer is more and more frequently forcing

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to the use of milk that has been subjected to the heating process misnamed "pasteurization." The point referred to is, "What is the effect of heated milk on a mixture of various varieties and numbers of those organisms commonly inhabiting milk and whose source is not only the cow but air, water, soil, dust, etc.

After commercial heating milk is almost invariably reinfected with a variety of organisms before it leaves the hands of the dealer,[16] and is subject to many sources of infection in the ordinary household before it is consumed. The work of isolating in pure culture the organisms inhabiting a number of specimens of milk is appreciated only by those who have tried it; and the returning of such cultures to the heated milk in their original quantity, quality, and relative proportions is a feat which has not been, and is not likely to be, readily accomplished. The addition of a portion of raw milk, with its accompanying organisms, to a portion of the same milk previously heated is open to objection because it either greatly reduces the actual number of organisms per c.c. which, according to Hunziker,[15] makes a difference in germicidal activity, or it carries in a goodly quantity of raw, unchanged milk which may modify the final result.

Because of the importance of the practical side of this question, that is, whether the commonly occurring mixtures of milk organisms show a different rate of growth in raw and in heated milk, we decided to devise a scheme by which the organisms, in like number and species and unchanged in activity by isolation on artificial media, could be studied side by side in the fresh, clean, raw product, and in the fresh, clean, pasteurized milk.

This we accomplished by centrifuging raw milk at a high rate of speed until the majority of the organisms had settled at the bottom of the tube. Plating the supernatant milk showed that from 50 to 75 per cent of them were thrown down after 15 minutes, whirling at a speed somewhat above 3,000 revolutions per minute. By carefully pipetting off the milk the sediment could all be kept in a volume less than 1 c.c. It was then transferred to milk which had been heated under the conditions desired. A sufficient amount of residue was added to supply the heated milk with approximately its original quota of organisms. Of course, due precautions were observed throughout to prevent contamination from handling.

That the reinfection gives a milk bacteriologically comparable with the raw product is proven not only by the total initial count, but by the proportions of acid-formers and liquefiers present in both samples, which, as the tables show, are practically identical.

The heating of the milk was carried out in small flasks at a temperature of 79° C. for 20 minutes, cooling quickly in running water and finally on ice. It will be noticed that the germs surviving were very few. As soon as it was cold it was reinfected and both samples were then plated.

The counts were made on plain agar, litmus-lactose agar, and litmus-lactose gelatin. The gelatin plates, showing the liquefying species as well as the acid-formers and general count, were, of course, kept at 20° C. The agar plates were grown for 24 hours at 37° C., then for two days at room temperatures. We find this initial high temperature gives clear-cut colonies, an especially desirable condition when acid-formers are to be counted.

The three samples to be compared, (*a*) raw, (*b*) heated and reinfected, (*c*) heated, were then kept either at room or refrigerator temperatures and plated at intervals of 24 hours until curding took place.

This happened, ordinarily, in from 72 to 96 hours for the pasteurized-reinfected milk, at which time the raw milk was still sweet and remained so for at least 24 hours longer, making the life of the raw milk, as determined by curding, considerably longer than that of the pateurized-reinfected milk. While the life of all the samples at room temperature was shorter than when kept in the ice-box, the relative differences were still well marked.

Counts made of these samples as above outlined showed a very marked increase in the bacterial content of the pasteurized-reinfected samples as compared with the raw. This increase ordinarily was plainly visible at the end of the first 24 hours, though if kept very cold there was an occasional decrease for that length of time, which decrease was more plainly marked in the raw than in the heated sample. In one case only, out of all the experiments made, was this decrease maintained for a longer period (Exp. 5, Table 1), and the whole course of this milk in all three samples differs so widely from the rest that too much stress cannot be laid upon it. It is given.

principally, to show what wide variations may occur in individual cases.

The decrease during the first day in these experiments is of additional interest because the milk was between six and eight hours old when we received it and its ability to kill organisms is, therefore, of fairly long duration. Prior to its delivery at the laboratory, however, its history was one of most unusual cleanliness and care. It was drawn from cattle inspected monthly by a veterinarian, tuberculin tested twice yearly; each individual cow was examined weekly for evidences of udder inflammation, as shown by the presence of pus or streptococci or staphylococci in quantity in her milk. The most rigorous practices regarding cleanliness of barns and cattle are followed, including a separate room for milking. The cooling of the milk is to below 40° F., generally 34° to 36°, and is accomplished so promptly that scarcely five minutes elapse between the drawing of the milk and its final sealing in sterile bottles, with sterile caps made flush with the bottle top with paraffin to prevent contamination while in transit to the consumer. The milk was packed in ice for transportation. If a germicidal property is an attribute of *clean* milk, as Hunziker[15] states, it should certainly find expression in milk so produced.

If, on the other hand, as Stocking[10a] asserts, "The decrease in the numbers of the normal milk bacteria during the first few hours after milking is not properly to be attributed to a germicidal condition or property possessed by the milk, but simply to the natural dropping-out of those species which do not find the milk a suitable medium in which to develop," the returning to the heated milk of the unchanged organisms should still be followed by the same decrease, provided the heating has in no wise changed its behavior as a culture medium.

Aside from this decrease in the first few hours, however, there remains the question of the relative rate of increase of the organisms in the raw and heated milk, a question which, as we said before, is of vital interest when considered from the standpoint of infant-feeding.

Table 1 gives five experiments made as above outlined, the results being expressed as ratios of increase, taking the initial count as unity. Accordingly, we find, in Exp. 1 that after 48 hours at room tempera-

ture the raw milk has increased its original content 1,355 times; the pasteurized milk has 51,904 times as many as in the beginning. In Exp. 3, after 96 hours in the ice-box, the raw milk has multiplied 1,328 times; while the pasteurized and reinfected has 34,550 times as many as in the beginning.

TABLE 1.

RATIO OF INCREASE OF GERM CONTENT.

	Number of Hours	Raw Milk	Pasteurized and Reinfected Milk
Experiment 1— Room temperature	Immediately	1:1	1:1
	24 hours	1:29	1:159
	48 "	1:1355	1:51,904
Experiment 2— Room temperature	Immediately	1:1	1:1
	24 hours	1:7	1:67
	48 "	1:55	1:35,000
Experiment 3— Ice-box temperature	Immediately	1:	1:
	24 hours	1:4	1:15
	48 "	1:218	1:637
	72 "	1:967	1:2700
	96 "	1:1328	1:34,550
Experiment 4— Ice-box temperature	Immediately	1:	1:
	24 hours	Loss of organisms	Decrease in count
	48 "	1:1.1	1:2
	72 "	1:3	1:12
	96 "	1:8	1:239
Experiment 5— Ice-box temperature	Immediately	1	1:
	24 hours	Decreasing count for 24 hrs.	Decreasing count for 48 hrs.
	48 "	1:1.8	
	72 "	1:1.3	1:1.6
	96 "	1:2.5	1:6
	120 "	1:22	1:84
	144 "	1:222	1:1703
	168 "	Sour	1:2222

Experiment 6 gives in greater detail the work leading to such figures as are summarized in Table 1. In this case the acid colonies were differentiated by counting on litmus-lactose agar and their record appears in the specified column. The milk was kept at 15° C. during the experiment.

Experiment 7 shows not only the course of the acid-formers, but also those organisms liquefying gelatin, a group of great importance in heated milk. The temperature here was 15° C.

Both of these experiments follow the usual course—a marked increase in the number of organisms in the heated milk. This is not, apparently, confined sharply to either the acid-forming or peptonizing groups, sometimes one, sometimes the other being in excess. However, at the close of the experiments, the peptonizing organisms are generally ahead in the heated milk.

EXPERIMENT 6.

Raw Clean Milk					Pasteurized Clean Milk Reinfected				Pasteurized Milk
Number of Hours	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Organisms per c.c.
Immediate.....	7,500	1:1	3,500 46%	1:1	7,200	1:1	2,600 36%	1:1	10
24 hours.....	51,000	1:6.6	13,000 25%	1:3.6	130,000	1:18	55,000 42%	1:21	..
48 "	2,000,000	1:266	500,000 25%	1:142	3,600,000	1:500	500,000 14%	1:192	..
72 "	288,000,000	1:38,400	84,000,000 29%	1:24,000	460,000,000	1:63,888	94,000,000 20%	1:36,153	..

EXPERIMENT 7.

Raw Clean Milk							Pasteurized Clean Milk Reinfected					
Number of Hours	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Liquefiers per c.c.	Ratio of Increase	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Liquefiers per c.c.	Ratio of Increase
Immediate...	1,370	1:1	650 47.5%	1:1	150 10.9%	1:1	1,250	1:1	540 43.2%	1:1	150 12%	1:1
24 hours.....	2,300	1:1.6	650 28.2%	1:1	200 8.65%	1:1.3	2,200	1:1.7	200 9.0%		600 27%	1:4
48 "	210,000	1:91	82,000 39%	1:126	64,000 30%	1:426	514,000	1:411	350,000 68%	1:648	200,000 46%	1:2,933
72 "	5,900,000	1:4,304	104,000 1.7%	1:601			11,700,000	1:9,360	660,000 5.5%	1:1,222		

EXPERIMENT 8.

Number of Hours	Raw Clean Milk 80 Hours Old: Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers	Ratio of Increase	Total Number Liquefiers per c.c.	Ratio of Increase	Pasteurized Clean Milk Reinfected 80 Hours Old: Sediment Washed: Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Liquefiers per c.c.	Ratio of Increase	Pasteurized: Number of Organisms per c.c.
Immediate..	80,000	1:1	13,000 16%	1:1	2,400 3%	1:1	10,000	1:1	4,100 41%	1:1	300 3%	1:1	20
24 hours....	7,600,000	1:95	540,000 7%	1:41	100,000 1.3%	1:41	7,050,000	1:705	2,100,000 29%	1:512	110,000 1.5%	1:366	..
48 "	25,800,000	1:322	8,600,000 33%	1:661	6,000,000 23%	1:2,500	28,000,000	1:2,800	13,000,000 46%	1:3,170	3,000,000 10%	1:13,333	..
72 "	264,000,000	1:3,300	80,000,000 30%	1:6,153	8,000,000 3%	1:3,333	257,000,000	1:25,700	55,000,000 21%	1:13,414	7,000,000 2.7%	1:23,000	..
96 "	42,000,000	1:525	11,000,000 26%	1:846	20,000,000 47%	1:8,333	67,000,000	1:6,700	12,000,000 17%	1:2,926	35,000,000 52%	1:116,666	..

MILK FROM MILK SHOP—NO HISTORY

	Raw Milk						Pasteurized Milk Reinfected						Pasteurized Milk
	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Liquefiers per c.c.	Ratio of Increase	Total Number of Organisms per c.c.	Ratio of Increase	Total Number of Acid-Formers per c.c.	Ratio of Increase	Total Number of Liquefiers per c.c.	Ratio of Increase	Total Number of Organisms per c.c.
Exp. 9													
Immediate..	23,000	1:1	8,400	1:1	5,000	1:1	17,000	1:1	5,000	1:1	4,000	1:1	40
			36%		21.3%				35%		23%		
24 hours....	1,620,000	1:70	.....	....	600,000	1:120	2,320,000	1:136	.....		800,000	1:200	..
					37%						38%		
48 "	10,000,000	1:434	4,600,000	1:547	5,000,000	1:1,000	3,200,000	1:247	1,200,000	1:240	1,800,000	1:450	..
			46%		50%				37.5%		56%		
Exp. 10													
Immediate..	500,000	1:1	200,000	1:1	400,000	1:1	300,000	1:1	120,000	1:1	40,000	1:1	..
			40%		80%				40%		13%		
24 hours....	107,000,000	1:214	82,000,000	1:410	45,000,000	1:112	100,000,000	1:333	50,000,000	1:416	30,000,000		
			76%		42%				50%		30%		
48 "	Sour						Sour						

Centrifuging and returning the sediment to pasteurized milk does, of course, enrich the milk in serum to some extent. Lest such an addition should affect in any way the growth of the bacteria we washed the sediment in sterile physiological salt solution several times before sowing the milk to be replenished—thereby removing this factor, if it be one. The experiment is outlined in No. 8. This milk also differed from the others in that it was 80 hours old when used, having been kept on ice to see whether the age factor, after the first few hours, made any great difference in the results obtained.

Apparently the amount of serum which this method adds to the milk does not alter the relative rate of growth of the organisms; for, though the initial count in the reinfected milk is lower than in the raw, due, of course, to the difficulty of washing and preserving the number of organisms intact, the ratio of increase in the heated milk is far in excess of the unheated. Originally, however, this milk was very clean and the temperature was low throughout.

What the effect of heat would be on an ordinary shop milk was the next question raised. But two experiments have been made, and they are not sufficient to give an adequate reply to the above query. The results are appended and we plan further work along this line with ordinary milk for which a history can be obtained.

CONCLUSIONS.

The foregoing experiments would indicate that aside from the lowering of the numbers of organisms in milk during the first few hours, be that due to a germicidal activity, or to unnatural environment, there is a restraining power for ordinary milk organisms in raw milk lasting at least to the curding point. This condition is either not present or is reduced in milk which is heated to 79° C., at a temperature below that required to destroy its oxydizing action.

This restraining power seems to apply to the organisms commonly found in milk in the combinations ordinarily present there. A complete qualitative study of the flora inhabiting the samples of milk cited was not carried through, but the predominating and most commonly occurring forms were isolated and proven to be *Bact. aerogenes*, *B. solitarius* (Ravenel), *B. formosus* (Ravenel), *M.*

aurantiacus, *Bact. fulvum* (Zimmermann), *M. viticulosis*, and *M. lactis*.

It is an accepted fact by many doing laboratory work on milk that pasteurization or sterilization of milk and its subsequent reinfection may be attended by serious consequences in the child, and, therefore, it is urged that even greater care in handling and refrigeration be employed for heated milk than for the raw article.

Such precautions, so far as a clean milk is concerned, would seem to be strongly emphasized by the foregoing experiments. They afford additional light, also, on the milk "pasteurized" commercially, which is reinfected after heating and generally, in cities, kept the better part of 24 hours before delivery to the consumer, who uses it at intervals during another 24 hours and who may heat it a second time.

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EPIZOOTIC LYMPHADENITIS.*

A NEW DISEASE OF GUINEA-PIGS.

CHARLES H. BOXMEYER.

THIS disease of guinea-pigs otherwise known as "lumps" is characterized by swelling of the lymph glands (most frequently the anterior cervical group) which subsequently break down with the formation of large abscesses. These abscesses on attaining their full size perforate the overlying skin and discharge a large quantity of cheesy pus. The number of abscesses varies from one to ten or twelve. They are usually in different stages of development. Marked constitutional symptoms are absent. Death results from a terminal systemic infection or from pressure causes.

History.—The outbreak here described occurred in guinea-pigs reserved for breeding purposes in a caviery belonging to a western antitoxin manufacturer. Some 3,000 were in the loft, over half of which showed the disease when examined in February, 1904. Cases of "lumps" were first observed two years before the above date and steadily increased in numbers. At least 3,000 animals have been attacked.

How the disease first originated is unknown. Attempts to trace it to outside sources failed. It has since been controlled by the slaughter of all infected pigs and by careful cleansing and fumigation of the caviery. Occasional cases still appear, however, as many animals carry internal abscesses that escape detection.

Morbid anatomy.—Autopsies have been made on over 100 pigs of all sizes, most of which were killed for examination. The following are the post-mortem findings in a few typical cases:

Guinea-pig No. 090. Red and white male; weight, 600 grams; found dead July 10, 1905.

Three of the anterior cervical glands are transformed into abscesses 15, 20, 50 mm. in diameter respectively. These abscesses are but loosely attached to the adjacent structures. Each is inclosed in a thick capsule over which run dilated blood vessels. All are filled with a thick cheesy pus, yellowish white in color. The pleural cavity is filled with a thick opalescent fluid. Fibrin is deposited on the visceral pleura.

* Received for publication June 16, 1904.

Lungs compressed and congested.

A tumor originating in one of the mediastinal glands displaces the lungs downward and the heart to the left.

Pericardium is greatly distended by thick fluid containing large white flakes.

Liver congested. It shows subcapsular hemorrhages and nutmeg markings.

Spleen slightly enlarged. Kidneys show acute parenchymatous changes. The abdominal lymph glands are but slightly enlarged.

Guinea-pig No. 010. Female, weight 300 grams. Chloroformed August 5, 1905.

A cluster of four cherry-sized abscesses in the anterior cervical region. A large egg-sized cheesy gland in the left axilla.

Uterus shows in the left cornu a cherry-sized abscess. Other viscera normal.

Guinea-pig No. 030. Female, weight 500 grams. Chloroformed June 14, 1905.

Submental glands. Three enlarged to the size of beans. On section they show a moist glistening surface with several pin-head areas of softening at the periphery of the gland. In the left anterior cervical region is a 30 mm. incapsulated abscess. In front of this is a pear-shaped tumor (15×30 mm.) containing pus. The deep cervical glands are much enlarged and edematous. Viscera normal.

The neck glands are the most often affected, the anterior cervical, submental, or auricular glands in over 90 per cent of the animals examined. Next in frequency the axillary glands are involved, then the groin. Abscesses arising from a retro-peritoneal gland near the sacro-vertebral articulation are frequent. Large abscesses 30 mm. in diameter are found here closing the pelvic inlet. Abscess of the spleen was observed five times. In one case two abscesses were present, one pointing in the lateral abdominal wall. Abscesses of the mediastinal glands were found three times. The liver, the uterus, and the lungs were each involved once.

The development of the abscesses can best be followed by studying animals of different ages, since the infection takes place early in life and the tumors reach their full development in adults.

The cervical lymph glands of normal guinea-pigs are not palpable. In infected pigs, at the age of three or four weeks, the glands have swollen to the size of small peas and may be plainly felt as hard nodules beneath the skin of the neck. On autopsy the swollen edematous glands are seen lying in the more or less congested and edematous fascia. The glands show, on section, a moist, uniformly white surface. Areas of softening are not yet present.

In pigs weighing 250 to 300 grams the glands have reached the size of large beans and are for the greater part bean-shaped. Inflammatory changes in the surrounding tissue are not marked. On section

the diseased glands appear much as in the smaller pigs, but in many, several small areas of softening are to be seen in the cortex.

FIG. 1.—Photograph of Guinea-pig No. 090. Kaiserling preparation. *a*, Mediastinal gland; *b* heart; *c*, abscesses arising from cervical glands.

In older pigs these areas of softening increase in size and fuse. They become surrounded by a firm capsule. But one end of the

gland may be broken down at this time into cheesy pus. From now on the growth of the tumors is rapid, the entire gland softens, the capsule thickens and becomes distended with pus. Large blood vessels are formed in the capsule and branch over its surface. The abscesses may reach the size of a hen's egg before rupturing through the skin. When the skin is perforated the pus is discharged and the abscess slowly heals by granulation. The pus is yellowish white and of the consistency of *Schmierkäse.*

Pigs dead of the disease show a general peritonitis, purulent pericarditis or pleuritis, or a pyemia.

Microscopic anatomy.—The lymph glands of pigs naturally infected or of inoculated animals which do not die acutely, show in the earliest stages of disease a marked dilation of the lymph sinuses and a desquamation of their endothelial cells. These cells have a ragged outline and show degenerative changes. The nucleus contains but very little chromatin and the cytoplasm stains faintly pink with eosin. These cells tend to collect in the cortical sinuses where they often fuse to multinucleated masses of considerable size.

While the germ centers show active proliferation, there is a marked decrease in the number of lymphocytes.

The capsule of the gland shows local proliferation. The peripheral lymph sinus is obliterated in these places and fibroblasts invade the cortex of the gland. At the same time, there is a development of large endothelial cells in the cortex and these with the fibroblasts form dense masses of large mononuclear cells. These cell groups form first, as a rule, in the spaces between the follicles of the gland involving them as they increase in size. The cytoplasm of the cells forming these masses stains deeply at first with basic dyes, but rapidly loses this power and acquires a slight acidophile character. The cells have large vesicular nuclei with small chromatin contents. A growth of fibroblasts tends very early to surround the mononuclear cell masses which are multiple and scattered around the periphery of the gland. There is a marked increase in the size of the lymph nodes due to this cell increase.

In later stages of the disease, polynuclear leucocytes appear in the centers of the cell collections. At times they are taken up by the mononuclear cells which are often completely filled with them.

From now on there is a rapid growth of the glands due to mononuclear increase and polynuclear infiltration. The foci soften in the center and pus is formed as in ordinary abscesses. Meanwhile, the fibroblasts form a capsule of increasing thickness around them. As the areas of softening approach each other, their individual capsules fuse more or less completely; a common capsule forming around them. This capsule thickens and becomes a true pyogenic membrane.

Microscopically the pus consists of polynuclear leucocytes and large mononuclear cells in various stages of disintegration. The large mononuclears are frequently loaded with cocci. Dense masses of necrotic cells are seen in the fluid portion of the pus.

Bacteriology.—In smears made from pus of the abscesses Gram staining cocci are always found either free or within the cells. They are generally present in large numbers, though many do not stain well. These organisms are usually seen as diplococci or short chains of four to six members, again as dense clumps or single cocci. These same cocci can almost always be found in smears and section made from the glands that have not yet begun to soften.

A large number of cultures have been made from about 200 animals. In every case a streptococcus has been obtained, in most instances in pure culture. The only other organism found has been a member of the hog-cholera group.[1] This organism has appeared in only a few cases and in these lesions characteristic of this group of organisms were present in the liver and spleen.

Except for some variations in virulence and fermentative power the streptococcus has apparently always been the same. As the virulence of the organism increases it tends to form longer chains in the animal body. In the peritoneal exudates occurring in terminal infections and in abscesses formed in very young pigs following inoculation with pus or cultures, it occurs as chains of considerable length.

The streptococcus in cultures occurs as chains of from 4 to 30 or more cocci somewhat variable in size. It is non-motile. No capsule has been seen. It stains well by Gram's method and with the ordinary stains.

Broth.—The growth is not very profuse. At the end of 24 hours the broth is perfectly clear. A considerable amount of white coarsely granular sediment

[1] SMITH AND STEWART, *Jour. Boston Soc. Med. Sci.*, 1897, p. 12.

is seen in the bottom of the tube. This soon subsides after shaking the tube. Under the microscope the sediment is seen to consist of tangled chains of streptococci.

Glucose broth.—The growth is more luxuriant than in plain broth. In fermentation tubes the greater development takes place in the closed branch, the growth adhering to the glass in filamentous masses. Acid, but no gas is formed.

Agar.—On the surface the growth occurs as minute colorless, usually discrete, circular colonies with entire margins and raised convex surface. The colonies show finely granular under the microscope. In moist media, chains and loops of streptococci are seen running out from the margins of the colonies. The color is yellowish white by reflected light and brownish by transmitted light. As the colonies increase in size they flatten. Stick cultures show minute globular colonies along the line of puncture.

Blood agar.—The colonies are identical in appearance with those on ordinary agar. A clear zone due to hemolysis of the red corpuscles surrounds each colony. The hemoglobin is not changed.

Löffler's blood serum.—This is the most favorable medium. Growth shows as minute dew-drop colonies usually more or less discrete. In very moist media the colonies may attain a diameter of 3 mm. The condensation water is clouded and contains a flocculent sediment.

Potato.—Minute globular pearl-like colonies appear. The condensation water is clouded heavily. Involution forms with marked variation in the size of the individuals forming the chain are seen.

Litmus milk.—Is rapidly reddened. Coagulation does not occur spontaneously in 10 days, but follows on heating old cultures.

Glucose and maltose serum waters are coagulated in 24 hours.

Lactose serum water is reddened. But the acid production is less than in milk.

Mannite is not fermented.

Susceptible animals.—The streptococcus of "lumps" is pathogenic for guinea-pigs, rabbits, and mice. It has not been tested on other animals. Mice and rabbits are apparently not infected spontaneously.[1]

Very young guinea-pigs are most susceptible. They die in from 1 to 30 days after inoculation with pus or cultures, from septicemia or pyemia. There is a marked local reaction and a swelling of the lymph glands in the region of the inoculation.

Subcutaneous and intraperitoneal injections lead to death in from 24 to 48 hours. Older pigs occasionally react only locally to subcutaneous inoculation.

A young rabbit inoculated with a culture by rubbing on the abraded skin of the ear, died in four and a half days. Autopsy showed a moderate local reaction and swelling of the cervical glands. Strep-

[1] Abscesses similar in appearance to those in guinea-pigs have been occasionally encountered in rabbits, but these contain pus of a different character and differ from "lumps" in their microscopic anatomy. These have always yielded cultures of an organism of the *B. mucosus capsulatus* type.

tococci were recovered from the heart blood. An adult rabbit showed a slight local reaction to cutaneous inoculation.

Subcutaneous injection of 0.5 c.c. in one rabbit and 0.1 c.c. into the ear vein of another caused death.

Mice have died after either cutaneous or subcutaneous inoculation from general sepsis.

Mode of infection.—Infection takes place as a rule early in the life of the animal. Adult animals show always cheesy glands and never the incipient stages alone. In addition, adult pigs are much less susceptible to experimental infection than the young.

TABLE.

No. Pig	White Cells	Per Cent Polynuclear	Per Cent Lymphocytes	Per Cent Large Mononuclear			Per Cent Eosinophile	Per Cent Mast Cells	
				Non-granular	Granular	Vacuole			
8...	12,400	52.0	25.2	12.0	5.3	0.6	4.3	0.6	
7...	12,400	45.0	29.7	16.0	5.3	2.0	1.0	1.0	
6...	14,600	75.0	15.0	8.0	1.0	0.7	0	0.3	
5...	13,500	40.4	45.0	12.7	1.6	0.3	0	0	
4...	12,400	42.0	46.0	....	10.0	...	1.7	0.3	
3...	14,800	50.3	43.2	....	6.4	...	0.1	0	8 normoblasts
2...	13,600	35.1	53.5	....	9.1	...	2.3	0	
1...	25,200	61.3	21.3	....	14.0	...	2.7	0.4	1 neutrophile myelocyte
10...	12,800	32.4	46.0	....	8.0	...	13.3	0.3	
9...	20,700	43.3	47.4	....	8.3	...	0.7	0.3	

The disease is without doubt a wound infection spread by the pus that escapes when abscesses rupture externally. The virus enters through small abrasions in the throat, in most cases caused by the rough food, e. g., oats, hay, etc. In proof of this, six guinea-pigs, weighing 300 grams each, were fed oats inoculated with a culture of streptococci. All showed temporary swelling of the cervical lymph glands, and one developed typical abscesses. Typical cervical abscesses have been repeatedly produced by rubbing culture or pus on the abraded skin of the ear. A healthy female who ate her artificially infected young showed three days later swelling of the cervical glands and 30 days afterward five or six enlarged cheesy cervical glands and a bean-sized gland in the left groin. The habit of biting each others' ears, which guinea-pigs have, explains the involvement of the auricular glands.

Infection follows the lymph stream. Inoculation on the fore-

foot leads to abscess of the axillary glands; on the hind foot, to abscess of the groin glands; on the ear, to the auricular and cervical glands.

The foregoing table gives the result of the examination of the blood of 10 adult guinea-pigs with cheesy glands. The red cells are unchanged from the normal in number and appearance.

Burnett[1] gives the normal number of leucocytes as 10,897 on the average, and the polynuclears as 31.52 per cent.

The findings then show a moderate leucocytosis and polynucleosis —a condition to be expected in an infection of this kind.

[1] BURNETT. *Jour. Med. Res.*, 1904, 11, p. 537.

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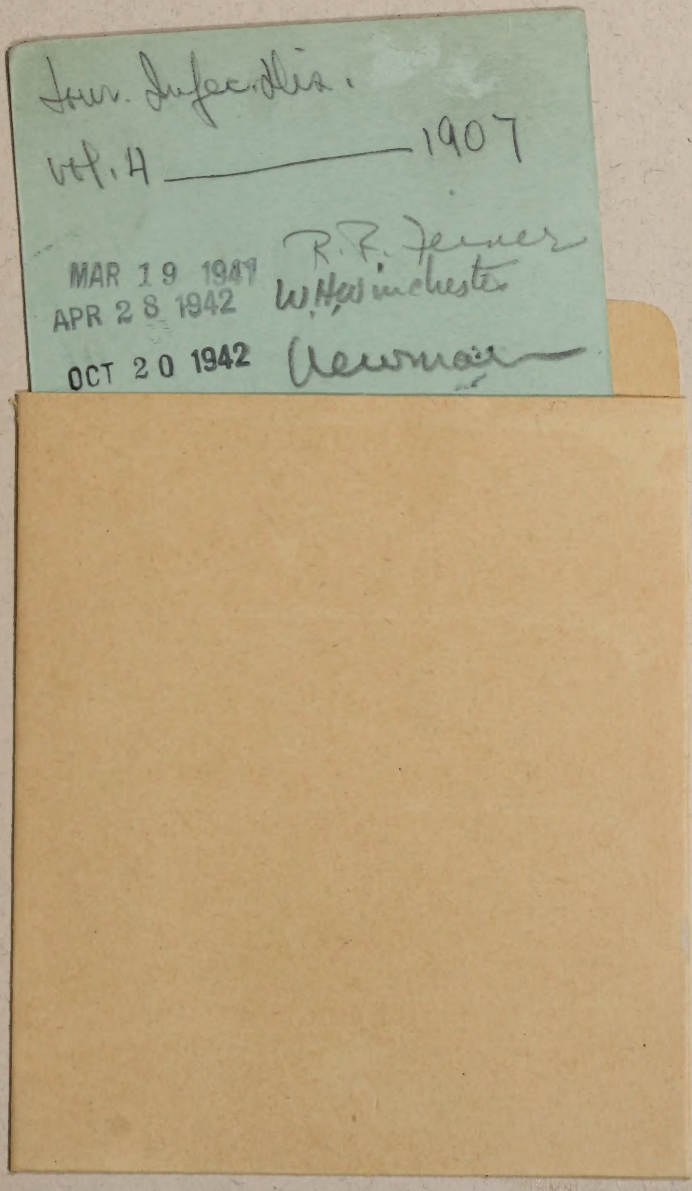

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